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Design of a tool to study the fate of *Chlamydia trachomatis* infected cells

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Resumo

Chlamydia trachomatis possui um ciclo de desenvolvimento intracelular único que termina pela lise da célula e/ou extrusão das bactérias a fim de permitir re-infecções. Esta é uma infeção maioritariamente assintomática, sendo o diagnóstico de *Chlamydia trachomatis* geralmente tardio, ou seja, após a manifestação de persistência. Investigações sobre as consequências destas infecções a longo prazo e dos mecanismos moleculares adjacentes irão elucidar de que forma as bactérias podem modular as funções da célula hospedeira e qual o destino final destas após uma infecção. Tais estudos podem ser grandemente facilitados se as células infectadas se tornarem permanentemente marcadas durante e após a infecção. Desta forma, este projeto tem como objetivo o desenvolvimento de uma nova ferramenta genética que torne possível a rotulagem permanente das células infectadas por *Chlamydia trachomatis*. O plano foi o de gerar uma estirpe de *Chlamydia trachomatis* que codifica uma recombinase, CRE, agregada a uma proteína efectora do sistema de secreção tipo 3 (T3SS) da *Chlamydia*. Após a translocação para a célula hospedeira, a enzima recombinante CRE, devido à sua função de recombinação local específica, irá activar um gene repórter contido no genoma da célula hospedeira. Para este fim, a linha celular repórter usada contém um gene membrane-tagged, tdTomato (mT), flanqueado por duas sequências LoxP, seguido por um gene GFP. A translocação da CRE recombinase para esta linha celular irá promover a recombinação dos locais LoxP através dos quais as células infectadas alteram de fluorescência vermelha para verde de uma forma irreversível. A execução bem-sucedida deste mecanismo permitirá estabelecer uma relação direta entre a infecção por *Chlamydia trachomatis* e o destino posterior da célula infectada.

Termos-chave: *Chlamydia trachomatis*, Sistema de Secreção tipo 3, TARP, Clonagem, Manipulação genética, Sistema de recombinação CRE/LoxP.

Abstract

Chlamydia trachomatis has a unique obligate intracellular developmental cycle that ends by the lysis of the cell and/or the extrusion of the bacteria in order to allow for re-infections. While *Chlamydia trachomatis* infections are often asymptomatic the diagnosis of *Chlamydia trachomatis* is usually late, occurring after manifestation of persistency. Investigations on the consequences of long-term infections and the molecular mechanisms behind it will reveal light to what extent bacteria can modulate host cell function and what the ultimate fate of host cells after clearance of an infection is. Such studies on the host cell fate could be greatly facilitated if the infected cells become permanently marked during and after the infection. Therefore, this project intends to develop a new genetic tool that would allow permanently labeling of *Chlamydia trachomatis* host cells. The plan was to generate a *Chlamydia trachomatis* strain that encodes a recombinant CRE recombinase, fused to a secretory effector function of the *Chlamydia* type 3 secretion system (T3SS). Upon translocation into the host cell, this recombinant CRE enzyme could then, owing to its site-specific recombination function, switch a reporter gene contained in the host cell genome. To this end, the reporter line carried a membrane-tagged tdTomato (mT) gene flanked by two LoxP sequences followed by a GFP gene. The translocation of the recombinant CRE recombinase into this cell line was designed to trigger the recombination of the LoxP sites whereby the cells would turn from red fluorescence to green as an irreversible label of the infected cells. Successful execution of this mechanism would allow to draw a direct link between *Chlamydia trachomatis* infection and the subsequent fate of the infected cell.

Keywords: *Chlamydia trachomatis*, Type 3 Secretion System, TARP, Cloning, Genetic Transformation, CRE/LoxP recombination system.

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List of Abbreviations

µg – microgram

µl – microliters

AA – aminoacids

APS – Ammonium Persulfate

CDS – Coding Sequence

Cm – centimeters

Ctr – *Chlamydia trachomatis*

Ctr D – *Chlamydia trachomatis* serovar D

Ctr L2 – *Chlamydia trachomatis* serovar L2

d - day

d.p.i. – day post infection

d.p.r.i. – day post-re-infection

dATP – deoxyadenosine triphosphate

DNA – deoxyribonucleic Acid

dTTP – thymidine triphosphate

E. coli – *Escherichia coli*

EB – elementary body

g – gram

h – hour

h.p.i. – hour post infection

IB – intermediate body

IFU – inclusion forming units

Inc – inclusion

LB – liquid broth

LSL – Lox-Stop-Lox

mA – milliamps

mG – tandem dimer GFP

min - minutes

ml – milliliter

mm – millimeters

MOI – multiplicity of infection

mT – tandem dimer Tomato

ng – nanogram

ºC – degree celsius

PCR – polymerase chain reaction

RB – reticulate body

rpm – rotations per minute

rcf – relative centrifugal force (g-force)

RT – room temperature

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC – super optimal broth

SPG – sucrose-phosphate-glutamic acid

T3S – type 3 secreted

T3SS – type 3 Secretion System

TARP – translocated actin recruit-protein

TEMED – *tetramethylethylenediamine*

U – units

V – volts

WT – wild type

I. Introduction

1. *Chlamydia spp.*

The family *Chlamydiaceae* is integrated by gram-negative bacteria that infect the ocular, genital and pulmonary surfaces. It is reunited into a single genus, *Chlamydia*, which involves nine species: Chlamydia *trachomatis, Chlamydia muridarum, Chlamydia pneumoniae, Chlamydia pecorum, Chlamydia suis, Chlamydia abortus, Chlamydia felis, Chlamydia caviae,* and *C. psittaci*. These species exhibit major differences in host range, tissue tropism, and disease pathology and affect a wide range of vertebral hosts, causing infections with serious impact on human health and on farming and veterinary industries (Agaisse and Derré, 2013; Betts-Hampikian and Fields, 2010; Bush and Everett, 2001; Nunes and Gomes, 2014). Species were grouped according to their biologic and biochemical properties, their homology in the 16s ribosomal RNA sequences and their gene for the major outer-membrane protein (MOMP) (Bush and Everett, 2001; Peeling and Brunham, 1996).

A. Pathogenesis and Biology of Chlamydia trachomatis

Ctr isolates are divided into, at least, fifteen serologically defined variants (serovars) which are associated with different pathologies, including blinding trachoma (serovars A–C), urogenital tract infections leading to urethritis, cervicitis, and proctitis (serovars D–K) and systemic lymphogranuloma venereum (LGV) disease (serovars L1, L2 and L3) (Bastidas *et al*., 2013; Bauler and Hackstadt, 2014; Wang *et al*., 2013a). *Ctr* is the most frequently reported cause of sexually transmitted disease worldwide, with an occurrence of approximately 100 million cases per year (WHO, 2011). The incidence of reported confirmed cases increased by 41%, between 2006 and 2010, in EU/EEA countries (ECDC, 2012). The most common serovar detected worldwide is E (up to 22 to 49% of cases) followed by serovars F and D (17 to 22% and 9 to 19%, respectively), while other serovars are less frequently identified (Papadogeorgakis *et al*., 2010). Like other sexually transmitted infections, it is primarily a women's care issue since the manifestations and consequences are more damaging to the reproductive health of women than men (Paavonen and Eggert-Kruse, 1999). One of the biggest complications is that, regardless of the gender, the majority of the infections, remain asymptomatic and difficult to diagnosed; hence bacteria persist for month, or even years. Persistence and recurrent infections with *Ctr* can often lead to infertility due to tubal occlusion and pelvic inflammatory diseases (Chin *et al*., 2012; ECDC, 2012; Kessler *et al*., 2012; Land *et al.*, 2010). In Europe and United States of America, *Ctr* infection is the main cause of tubal infertility, which increase directly proportional to the number of pelvic infections in the past (Wilkowska-Trojniel *et al.*, 2009).

It has been recently implied that chronic bacterial disease can have a direct role in carcinogenesis, in the case of *Chlamydia* several studies have been made in the intend to correlate this pathogen with cervical and ovarian cancer (Arnheim Dahlström *et al*., 2011; Koskela *et al*., 2000; Shanmughapriya *et al*., 2012).

Chlamydia developmental compromises a very complex cycle divided in two main forms (Figure I.1). The infection starts with the attachment of the elementary bodies (EBs) (the infectious and metabolically inactive 'spore-like' form of the bacteria used to extracellular survival) to the surface of the host cells. After attachment *Chlamydia* induces the Rho-GTPase Rac1 and the chlamydial protein TARP (Translocated Actin Recruitment Protein) is translocated to the host cell, resulting in an actin-rearrangement allowing the entry of the EBs. The arising phagocyte compartment is rapidly modified by proteins derived from *Chlamydia* generating a replicative niche termed inclusion (Bastidas *et al*., 2013; Bauler and Hackstadt, 2014; Valdivia, 2008). Within the inclusion the EBs that has a rigid cell wall conferred by extensive disulfide cross-linking of the major outer membrane protein (MOMP), differentiate into reticulate bodies (RBs), the active and replicative form of the bacteria. Afterwards, RBs undergo several replicative cycles within the inclusion that is intimately associated with recycling endosomes that translocate through a dynein-dependent mechanism to the peri-Golgi region and replicates by binary fission. During this stage, a subset of Rab GTPases are recruited to the inclusion membrane and the inclusion expands becoming the replication asynchronous in the differentiation from RBs back into the EBs form (Bastidas *et al*., 2013; Beatty *et al*., 1994; Engel, 2004; Valdivia, 2008; Wang *et al*., 2013a). Eventually most of the cytoplasmic space of the host cell is occupied by the inclusion and the newly formed EBs are further released from the host cell, by cell lysis (sequential disruption of the inclusion and cellular membranes by cysteine proteases) and/or extrusion (package release out of the cell within a cell membrane compartment, and ultimately detachment from the cell) (Hybiske and Stephens, 2007).

During the infection cycle on the host cell, when *Chlamydia* is subjected to stress (e.g. tryptophan starvation, exposed to beta lactam antibiotics) the developmental cycle slows and ceases to divide; also, the transition to EB becomes retarded. This results in aberrant RB formation where the RBs become enlarged and the developmental cycle enters into a persistent, non-infectious state. Like other stressed bacteria, these aberrant RBs express elevated levels of stress-response proteins and do not resume normal growth until the stressor is removed (Peters *et al*., 2010; Skilton *et al.*, 2009; Wang *et al.*, 2013a). In the specific case of penicillin treatment, this will not affect EB to RB differentiation but it will inhibit chromosomal condensation and, consequently, the transition from replicating RBs to infectious EBs in the later stages of the developmental cycle (Skilton *et al.*, 2009).

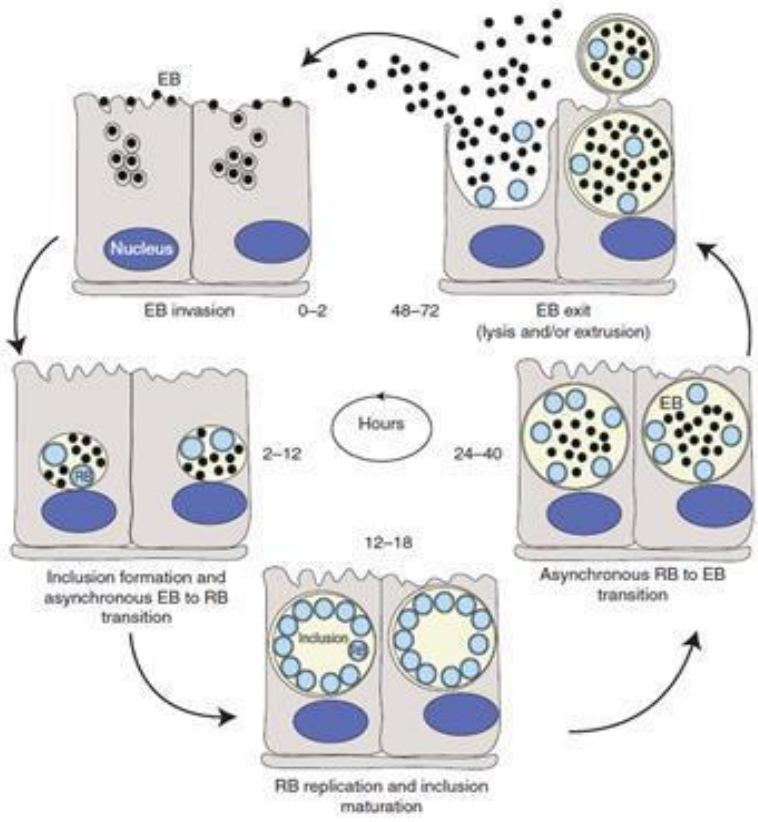

Figure I.1 Life cycle of *Chlamydia trachomatis*. Within the first 2 h following internalization into cells, elementary bodies (EBs) fuse to form a nascent inclusion. Between 2 h and 6 h post-internalization, EBs begin to differentiate into reticulate bodies (RBs). By 12 h post-infection (h.p.i.) RBs can be observed dividing by binary fission (18–24 h.p.i.). Increasing numbers of RBs differentiate back to EBs around 24 h.p.i. and continue differentiating until lysis and/or extrusion (48–72 h.p.i.) (Adapted from Bastidas *et al*. 2013).

B. Type 3 Secretion System

At both forms of the chlamydial cycle, effector proteins are translocated directly into the host cell for manipulation of diverse host-cellular functions, using a Type 3 Secretion System (T3SS) (Olive *et al*., 2014). The non-flagelar T3SS, Figure I.2, is composed by: Ancillary Components (AC) containing secretion signals and chaperons, essential for the translocation of the effector protein through the molecular syringe; the Basal Apparatus that is composed for different proteins that connect the outer and inner membrane of the inclusion. The exterior components are composed by the Needle Complex (NC), which consists of a membrane-embedded basal structure, that will connect the bacteria and the membrane of the host cell for translocation; the Tip Complex (TP) that only exist under non-secreting conditions and the Translocator proteins (Tr) which oligomerize to form a hetero-oligomeric pore in the host cell cytoplasmic membrane and the inclusion membrane (Betts-Hampikian and Fields, 2010; Chatterjee *et al*., 2013; Peters *et al*., 2010).

The T3SS system assembly is initiated by three structural proteins (CdsC, CdsJ and CdsD) that assemble the Basal Apparatus. As soon this structure is completed, components can be exported to form the NC that protrudes from the bacterial surface, and the TC that caps the needle (Betts-Hampikian and Fields, 2010).

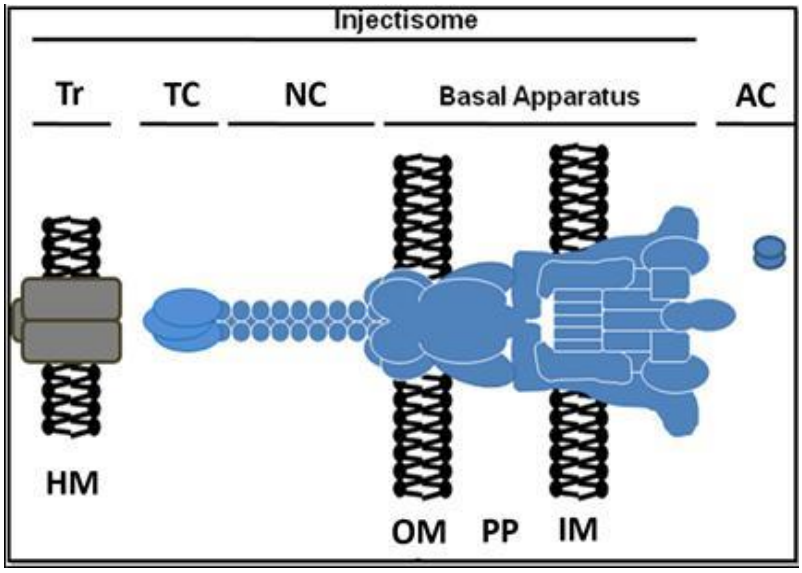

Figure I.2 Schematic representation of a Type 3 secretion injectisome. Components include: the translocator (Tr), tip complex (TC), needle complex (NC), basal apparatus, and cytoplasmic ancillary proteins (AC). The basal apparatus is within the bacterial inner membrane (IM), the periplasm (PP) and outer membrane (OM). Secreted components are shown localized to a host membrane (HM) (Betts-Hampikian and Fields, 2010).

C. Chamydial effectors

The knowledge of the cellular processes that are targeted by Ctr has greatly increased over the past 10-15 years, but only recently the effort to identify the host and bacterial factors required for bacterial development had begun (Derré et al., 2011). Numerous *Ctr* T3S substrates, between other features of *Ctr* have been found by using *Salmonella*, *Shigella* or *Yersinia* as heterologous hosts, or suggested by machine learning algorithms based on the N-terminal T3S signal of known effectors (Pais et al., 2013).

It is estimated that Ctr genomes may encode approximately one-hundred T3S substrates (Pais et al., 2013). T3S substrates display little sequence homology, although they often display common structural features and have similar enzymatic activities across bacterial strains. These proteins are either secreted into the inclusion lumen, deposited in the inclusion membrane or translocated directly to the cytosol of the host cell (Peters *et al*., 2010).

The *Chlamydia* inclusion contains 50 membrane proteins, including a number of proteins that show no sequence similarities but have in common the presence of a large (40±70 amino acids) hydrophobic region and have therefore been grouped into a structural family called the Inc family (Derré *et al.*, 2011; Subtil *et al*., 2001). These proteins are localized at the inclusion membrane and are known to be produced early in the chlamydial infectious process and consequently are candidates for initial pathogen-host cell interactions that lead to the formation of the inclusion (Bastidas *et al*., 2013; Rockey *et al*., 2002). The inclusion proteins identified go from IncA to IncG and only a few of them have established functions; for example, IncA, IncB and IncC are consider as probably the major components of the inclusion, with functions likely involved in the homotypic fusion of the *Ctr* inclusions (Derré *et al*., 2011; Subtil *et al.*, 2001).

Between the translocated substrates, in 2004, Clifton and coworkers reported that on entry, *Ctr* translocate an actin-recruiting protein (TARP) into the host cell, that corresponds to the chlamydial ORF CT456, which encodes a 103-kDa protein (Clifton *et al*., 2004; Engel, 2004). TARP protein becomes tyrosine phosphorylated by a host cell kinase, and is spatially and temporally associated with the recruitment of actin to the site of EB invasion, being considered an immediate early effector involved in the invasion and re-organization of the host cell cytoskeleton (Brinkworth *et al*., 2011; Clifton *et al*., 2004; Pais *et al.,* 2013). TARP is present in all pathogenic *Chlamydiae* examined to date, but only in *Ctr* serovars is phosphorylated, ranging the number of phosphorylation sites between serovars (Clifton *et al*., 2004; Engel, 2004; Jewett *et al*., 2008, 2006). TARP is directly associated with actin, and this interaction promotes actin nucleation and recruitment, initiating the remodeling of the actin cytoskeleton. This remodeling can be done by stimulating cell signaling, activating the host actin-nucleating Arp2/3 complex, which is crucial for *Ctr* survival on the host (Brinkworth *et al*., 2011). TARP also seems to mediate the resistance to pro-apoptotic stimuli upon host cells during the early stages of infection (Mehlitz *et al*., 2010).

The secretion of some T3S chlamydial substrates requires, in the bacterial cytosol, the assistance of chaperon proteins. Although various chlamydial substrates are reported, not so many T3S chaperones that bind to the effectors are. Recognition by the chaperons is done through a secretion signal—a short sequence of amino acids located at the beginning (the N-terminus) of the protein (usually within the first 50 amino acids), that the needle complex is able to recognize (Löwer and Schneider, 2009). Chaperon proteins stabilize the chlamydial effectors maintaining the effectors in a translocation-competent conformation to pass through the T3SS. In 2011, Brinkworth and colleagues reported that Slc1 (CT043) is the chaperon for TARP and based on the information reported by Clifton et al, the first 200 aminoacids in the N-terminal part of TARP are enough for secretion being consider the binding site for the chaperon (Brinkworth et al., 2011; Clifton et al., 2005, 2004). In 2013, Pais and colleagues, reported that Slc1 is also the chaperon of CT694 and CT695 and that the first 100 aminoacids of N-terminal region of TARP are necessary and sufficient for the binding of TARP to the chaperon (Pais et al., 2013).

D. Chlamydial Genome

Chlamydia trachomatis has a gene expression program consisting of early-genes (1-8h), mid-genes (8-16h) and late-genes (16-24h) which coordinate the transition between the chlamydial developmental forms and the synthesis of virulence factors (Valdivia, 2008) . *Ctr* has a genome that consists of 1,042,519 nucleotide base pairs and has approximately 894 likely protein coding sequences (CDS) (Stephens *et al.*, 1998). Most isolates of *Ctr* carry an extrachromosomal 7.5 kb plasmid which encodes eight CDS, arranged in a way that all but one of the genes are transcribed in the same direction. Assignment of biological functions has not been successful due to the absence of a simple mean to manipulate the chlamydial genome. Nonetheless, bioinformatic analyses have indicated that CDS1–CDS3 are probably involved in plasmid replication; CDS3 may be involved in encoding an enzyme involved in unwinding DNA during replication; CDS7 and CDS8 display sequence homology with other plasmid partition proteins and thus are likely to be essential for plasmid maintenance (Wang *et al*., 2013a, 2011).

Regarding the T3SS, 5-8% of chlamydial genome encodes targets of TTS and the genes are grouped in at least 4, and probably more, subclusters (Subtil *et al*., 2000; Valdivia, 2008).

E. Chlamydia-host interactions

Due to the small size of chlamydial genomes (<2Mb) with few repetitive elements and pseudogenes; many of the genes required for the biosynthesis of key metabolites have been lost and these are directly acquired from the host cell. This shows a strict dependence on the host for survival. These mechanisms allow the import and incorporation of nucleotides, amino-acids, lipids, iron and other essential host-derived nutrients from the host cell, while limiting detection by the innate immune system (Bastidas *et al*., 2013; Saka and Valdivia, 2010). It was also described that integration with multivesicular bodies (MVB), the Golgi apparatus, mitochondria and lipid droplets are required for optimal chlamydial replication (Saka and Valdivia, 2010).

Due to the importance of the host survival, *Chlamydia* prevents host cell apoptosis through a variety of mechanisms (Mehlitz *et al.*, 2010). *Chlamydia* uses a wide range of strategies that may subvert the host-immune responses, by interfering with the function of transcription factor NF-$_{\kappa}B$ (a central regulator of immune response), blocking the phosphorylation/ubiquitination/degradation of IkB so the NF-$_{\kappa}B$ does not go into the nucleus in order to block the expression of hundreds of genes, including innate immune response factors (Lad *et al*., 2007). *Chlamydiae* has also developed a mechanism to limit the recognition of PAMPs (Pathogen-associated molecular patterns) by ensuring the stability of the inclusion during the intracellular stages of infection (Kumar and Valdivia, 2008). Also, *Chlamydiae* infection leads to stabilization of IAP2 (inhibitor of apoptosis protein 2) and up-regulation of the pro-survival factor Mcl-1 (myeloid cell leukemia) (Rajalingam *et al*., 2008, 2006; Sharma *et al*., 2011). Infections can also activate MAPK (Mitogen-activated protein kinases) and PI3K (Phosphoinositide 3-kinase) pro-survival signaling

pathways which affect the bacterial nutrient acquisition, expression of antibiotic factors and synthesis of pro-inflammatory cytokines, such as the production of the inflammatory chemokine IL-8 (Interleukin-8) (Buchholz and Stephens, 2007; Rajalingam *et al*., 2008; Su *et al*., 2004). Moreover, *Chlamydiae* escapes the host defense mechanisms by preventing the fusion of the inclusion with acidic degradative compartments of the host cell, the fusion with lysosomes (Moulder, 1997; Subtil *et al*., 2001). Hackstadt and colleagues proposed that *Chlamydiae* meet these requirements by removing themselves from the endocytic pathway, by actively modifying the vacuole to become fusogenic with sphingomyelin-containing exocytic vesicles and not with lysosomes. Otherwise, that will lead to their eventual destruction in secondary lysosomes (Hackstadt *et al*., 1997). All these characteristics, among others, have a major impact on the survival of the host cell by avoidance of cell death and promoting self-survival (Bastidas *et al*., 2013).

2. Chlamydia trachomatis genome manipulation

The discovery of the DNA structure, its role and, as well, the knowledge of the genetic code had lead the discovery and efforts to the development of diverse genetic tools to work and handle DNA. This expertise is essential for a whole range of scientific and technological studies (Lodge *et al*., 2007).

Ctr was for a long time reported as refractory to transformation and although the very limited genetic exchange that *Chlamydia* goes, there was also a huge lack of genetic approaches to manipulate the genome of this bacteria (Bastidas *et al*., 2013; Mehlitz *et al*., 2010; Skilton *et al*., 2009; Subtil *et al*., 2001, 2000; Wang *et al*., 2013a). In 2009, an approach using chromosomal integration was used to make recombinants in *Chlamydia psittaci* EBs by allelic exchange using exogenous DNA introduced by electroporation (Binet and Maurelli, 2009). In 2011 some developments were made to mutate the chlamydial chromosome (Kari *et al*., 2011). Nonetheless a demand for development of a simple robust genetic transformation system for the C*hlamydiae* has remained a significant challenge (Wang *et al*., 2011).

In 2011, Wang and coworkers reported for the first time a successful, simple and robust plasmid-based genetic transformation system for *Chlamydia trachomatis* serovar L2 (*Ctr* L2) using penicillin selection and calcium chloride ($CaCl_2$) treatment of EBs. Wang and colleagues were able to produce a strain of *Ctr* L2 that is penicillin resistant and shows similar growth characteristics and inclusion formation as the WT (Wild Type) strain (Wang *et al*., 2011).

The ability to genetically manipulate *Chlamydiae* is a complementary advancement that will enhance the understanding of chlamydial pathogenesis; accelerating the development of new anti-

chlamydial therapeutic control measures, chlamydial vaccines and therapeutic interventions (Kari *et al*., 2011; Wang *et al*., 2011).

3. Genetic lineage tracing mediated by CRE recombinase

There are increasing evidences that some bacteria can contribute to specific stages in cancer development, particularly in chronic infections (Mager, 2006). These pathogens can be involved in the hypothesis that bacterial molecules are necessary for the initiation but not the maintenance of cellular transformation (Kuper *et al*., 2000). Therefore, identification of the damaged cells that have been in contact with the pathogen of interest could help to determine and describe the link between the pathogen and further transformations, like cancer (Lax and Thomas, 2002).

The cyclisation recombination (CRE) enzyme of bacteriophage P1, a 38 kDa site-specific recombinase was first developed as an *in vivo* molecular tool over two decades ago. CRE recombinase catalyzes recombination between its recognition sites (LoxP sites) in any cellular environment and on any kind of DNA. The LoxP sites consist of a consensus 34 base pairs DNA and a sequence of 8bp and 13bp palindromic flanking sequences. At least two LoxP sites are need for the CRE recombinase activity to occur (Guo *et al*., 1997; Nagy, 2000; Smith, 2011; Stricklett *et al*., 1999).

Inducible CRE cell lines are increasing in popularity due to the ability for elegant temporal, mutually exclusive and spatial targeting of genes. The development of CRE/LoxP has expanded and refined the toolbox of genetic manipulation available to researchers and there are now more than 500 independent CRE recombinase expressing mouse lines, many of which are accessed via CREXmice database (Muzumdar *et al*., 2007; Nagy *et al*., 2009; Smith, 2011). Since its initial use, several improved versions of CRE have been developed, which utilize eukaryotic codon preference to improve the expression of CRE, such as iCRE (Shimshek *et al*., 2002) or the addition of a eukaryotic nuclear localization signal to increase recombination efficiency (Kalderon *et al.*, 1984).

In 2007, Muzumdar and colleagues described a double fluorescent CRE recombinase reporter mouse. In this system (see Figure I.3) if CRE interacts with a mT/mG construct, that expresses tdTomato (mT) prior to CRE excision and membrane-target GFP (mG) after CRE excision, this will allow the live visualization and distinction of recombined and non-recombined cells, enabling bright fluorescent labeling of all tissues/cells: red before and green after CRE recombination. Additionally, the double fluorescent membrane targeted system permits tracing of lineage and characterization of cell morphology *in vivo* (Muzumdar *et al*., 2007). Livet and coworkers explored the advantages of the widely used CRE/LoxP

recombination system, which can switch on gene expression by DNA excision, inversion or interchromossomal recombination (Livet *et al*., 2007).

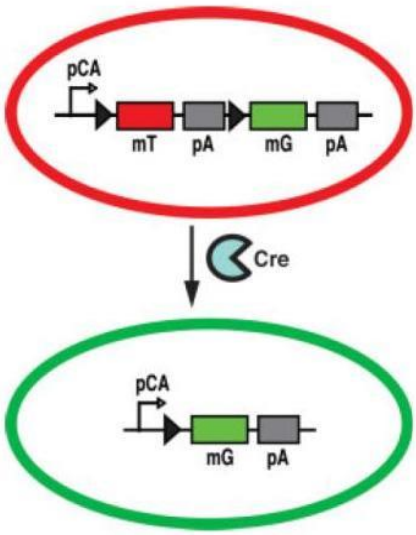

Figure I.3 The CRE/LoxP recombination system. Schematic example of CRE recombinase activity in a mT/mG reporter cell. mT/mG consists of a chicken bactin core promoter with a CMV enhancer (pCA) driving a loxP flanked coding sequence of membrane-targeted tandem dímer Tomato (mT) resulting in tdTomato expression with membrane localization. When CRE enters the reporter cell line the mT gene is excised due to CRE recombination activity and the reporter cell line becomes green instead of the red fluorescence. Arrows denote the direction of transcription. Triangles - LoxP target sites for Cre-mediated recombination. PA - polyadenylation sequences (Muzumdar *et al*., 2007).

Another modification of this system, described by Hirrlinger and coworkers is the introduction of a second dimension to cell-type specific control of DNA-recombination *in vivo*, the Split-CRE. They divided the CRE recombinase into two fragments and fused both to strong protein interaction domains. The resulting fusion proteins (NCRE and CCRE) were able to complement each other thus reconstituting functional CRE recombinase in neurons and glia of a living mouse. Split-CRE allows selective genetic targeting of cell populations defined by the simultaneous activity of two promoters in the mouse (Hirrlinger *et al*., 2009b).

The advantages of the CRE/LoxP recombination system are the no need for additional co-factors or sequence elements for efficient recombination regardless of the cellular environment. LoxP target sites are small and easily synthesized, there are no apparent external energy requirements and CRE is a stable protein (Nagy, 2000; Stricklett *et al*., 1999). Application of this technology to the mammalian system is possible because the mammalian genome lacks high affinity LoxP sites, therefore, artificially engineering these specific sequences into DNA regions flanking a target locus of interest for recombination by CRE

recombinase is a major gain to any genetic engineer system (Smith, 2011). CRE recombinase has several important applications as: (1) a traditional targeting and selectable marker removal; (2) CRE excision conditional gene knock-out; (3) CRE excision conditional gene repair; (4) it can be used as well in a combination with an inducible system by the use of tamoxifen, for instance; between many other promising applications (Nagy, 2000). As for Split-CRE, the complementation can be broadly applied and is independent of the expression method as well as the cellular context (Hirrlinger *et al*., 2009b).

Despite the enormous success of cell-specific gene targeting using Cre/LoxP, two major limitations are inherent to this system: (1) the time of recombination is solely determined by the time of onset of the promoter activity driving CRE expression and cannot be controlled experimentally; (2) the specificity of the promoter used to drive CRE expression is often not sufficient to selectively target a specific cell population (Hirrlinger *et al*., 2009a). On the other hand, CRE recombinase is certainly one of the key tools that made many of the newly developed genome alteration technologies possible (Nagy, 2000).

4. Aims

The aim of this project is to generate a genetically modified *Ctr* that is able to label the host cell following the infection. The label is permanent so that both, cells with ongoing infection and cells that survived after an infection, can be detected, isolated and characterized. With this tool is possible to monitor if cells are surviving the infection or not. Moreover, since it has been demonstrated that *Ctr* induces DNA damage in the host cell, this tool can be used to track cells that might have undergone cellular transformation directly triggered by the pathogen. This labeling system is based on a “signal” that is provided by the pathogen and on a “sensor” that is located in the host cell. The triggering “signal” is the CRE recombinase enzyme. CRE is bound to a translocated protein that is delivering the “signal” into the host cell. The “sensor” is based on a genetic reporter that changes permanently the expression of the fluorescent gene upon CRE recombination (red to green). In order to generate a genetically modified *Ctr*, a previously published transformation protocol was established.

Therefore, the present study was divided into three steps: (1) generation of a plasmid coding a chlamydial protein (TARP) fused with CRE for translocation via T3SS; (2) transformation of *Ctr* with the generated plasmid and (3) test the functionality of the system into a stable reporter cell line sensitive to CRE activity (HeLa229_mT/mG). This process should lead to an irreversible label that will allow the detection of cells which were at any time infected with *Ctr*. The establishment of such a system is a significant launch on further longer projects.

II. Materials

In Table II.1 to Table II.7 the reagents, solutions, antibodies, growth media, commercial kits, restriction enzymes and commercial plasmids, included in this study are enumerated by manufacturer and lot number.

Table II.1 Expendable reagents

Manufacturer	Reagents
Applichen	Tris ultrapure (Art. Nr: A1086.5000; Lot. 2Z002392); Bromophenol blue (Art. Nr: A233.0005; Lot. 9Q005789); Non-fat dried milk (Art. Nr: A08301000; Lot.20003941)
Bacto	Bacto yeast extract (Art. Nr: 212720; Lot.8023617)
Biochrom	Fetal Calf Serum - FCS (Art. Nr: S0115; Lot. 0710x)
Biomol	Albumin bovine (BSA) fraction V (Art. Nr: 01400.1; Lot. 1740); Glycine p.A. Aminoacetic acid (Art. Nr. 04943.2500: Lot.7844)
Carl Roth	NaOH (Art. Nr: P031.2; Lot. 2151855); X-gal (Art. Nr: 2315.3; Lot. 2306408); EDTA (Art. Nr: 8043.2; Lot. 2053583); Triton-X-100 (Art.Nr: 3051.2); Calcium Chloride (Art. Nr: A119.1; Lot.350158003); LB-Agar (Art. Nr: X965.2; Lot.250156129); X-Gal (Art. Nr: 2513.3); TEMED (Art. Nr: 2367.3; Lot. 2037446); Retiphorese Gel 30 (30% Acrylamid) (Art. Nr: 3029.1; Lot. 221172398); β-Mercaptoethanol (Art. Nr: 4227.1; Lot. 2004646); Glycerin (Art. Nr: 4043.3; Lot. 36790090); NaCl (Art. Nr: 9265.2; Lot.073196636)
Fisher Scientific	Ethidium bromide solution (Art. Nr: E/P800/10; Lot. 09255985); Tryptone (Art. Nr: BP1421500)
Gibco	DPBS (Art. Nr: 8043.2; Lot. 1479939/1525810/1532363); Trypsin (Art. Nr: 25300-096; Lot. 1401952/1508836/1550252); L-Glutamine (Art. Nr: 25030-024; Lot. 1437728)
Invitrogen	LB Broth Base (Art. Nr: 12780052; Lot.00810292)
Merck	Tween 20 (Art. Nr: 8.22184; Lot.); APS - Ammonium Persulfate (Art. Nr: K36015201707; Lot.1.01201.0500); Acetic Acid (Art. Nr: K40866663009; Lot.1.00063.2500); Methanol (Art. Nr: 1714009344; Lot.1.06009.2511); Isopropanol (Art. Nr: K43800534237; Lot.1.09634.2511); Ethanol (Art. Nr: K45251683403; Lot.1.00983.2511); Methylene Blue (C.I. 52015) (Art. Nr: 159270); Glucose (Art. Nr: K38076537806; Lot. 1.08337.1000); $MgSO_4$ (Art. N: 1.05886; Lot.A963086)

Sigma – Aldrich	Ampicillin (Art. Nr: A9518-1006; Lot. 074K0522); DAPI - bisBenzimide H33342 trihydrochioride (Art. Nr: B2261-100MG; Lot.116K4029); Mowiol (Art. Nr: 32,459-0); Paraformaldehyde (Art. Nr: 441244-1kg; Lot.MKBK8362V); Penicillin G + Sodium salt (Art. Nr: P3032-100MU; Lot.SLBC7349V); Tween-20 (Art. Nr: S6536884235; Lot.8.22184.0500); KCl (Art Nr: P9541); Cycloheximide (Art. Nr: C1988-1G; Lot.065K122261); Boric Acid (Art. Nr: B0252; Lot.57H0655); Magnesium chloride (Art. Nr: M2393-100G; Lot. 064K0110); Na-Pyruvate (Art. Nr: S8636; Lot. RNB07806)
Serva	SDS (Art. Nr: 20765.03; Lot.120895)
Thermo Scientific	Draq5 (5mM) (Art. Nr: 62252; Lot.316Dr51000): IPTG dioxiane free (Art. Nr: R0391; Lot. 99131); FastAP (Art. Nr: EF0654; Lot.00152428); FastAP Buffer 10x (Lot.00149725); 1kb DNA Ladder (Art. Nr: SM0311); 50bp DNA Ladder (Art. Nr: SM0371); Protein Ladder (Art. Nr: SM1812)

Table II.2 Solutions and Buffers

Solution/Media	Composition
Blocking Buffer for Immunofluorescence	BSA 0.5% (w/v), Tween 20 0.05%(w/v), PBS 1X
EDTA	0.5 M disodium ethylenediamine tetraacetate (pH8.0)
Lysis Buffer	25 mM EDTA, 5 mM Tris, 10% SDS, 20 mg/ml Proteinase k
SDS-PAGE Running Buffer (10x)	25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS
SDS-PAGE Sample buffer (2x)	62.5 mM Tris-HCl pH 6.8; 10% (w/v) Glycerol, 2.5% (w/v) SDS, 0.002% (w/v) Bromophenol blue.
SDS-PAGE Transfer Buffer	25 mM Tris-Base, 192 mM Glycine, 20% (w/v) methanol (pH 8.3)
SPG buffer (Sucrose-Phosphate-Glutamic acid)	220 mM Sucrose, 3 mM KH_2PO_4, 8 mM Na_2HPO_4, 5 mM L-Glutamic acid (pH 7.4)
TAE Buffer 50x	40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0)
TBE Buffer	89.15 mM Tris/HCl, 88.95 mM Boric acid, 2 mM EDTA
TBS 1x	50 mM Tris-Cl with pH 7.6, 150 mM NaCl
TE Buffer	10 mM Tris-Cl, 1 mM EDTA (pH 8.0)

Table II.3 Antibodies

Antigen	Specie	Dilution for IF	Dilution for WB	Company
Primary Antibodies				
Anti-*Chlamydia* MOMP	Mouse	1:10000	-	University of Washington (Lot.90813)
Anti-CRE recombinase	Mouse	-	1:1000	Abcam (Art N: Ab24607)
Anti-ß-Catenin	Rabbit	1:100	-	Sigma (Art N: C2206)
Secondary Antibodies				
ECL Anti-Mouse IgG	Sheep	-	1:3000	Amersham NA931-1ml (Lot. 9471753)
Cy2 Anti-Mouse IgG	Goat	1:200	-	Dianova (Lot.78409)
Cy2 Anti-Mouse IgG	Donkey	1:100	-	Dianova (Art. Nr: 715-225-151; Lot. 86595)
Cy2 Anti-Rabbit IgG	Goat	1:150	-	Dianova (Lot.81780)

Table II.4 Growth media and its composition

Manufacturer	Growth Media
Gibco	DMEM 1x (Art. Nr:10938-025; Lot.1450389/1482361/1485850)
	MEM 1x (Art. Nr:51200-046; Lot.1482357)
	RPMI 1640 1x (Art. Nr:52400-025; Lot.1437700/1528246/1437700)

Table II.5 Commercial kits

Manufacturer	Kit
Qiagen	QIAquick PCR Purification Kit (Art. Nr: 28106)
	QIAquick Gel Extraction Kit (Art. Nr: 28706)
	QIAprep Spin Miniprep Kit (Art. Nr: 27106)
	Qiagen Plasmid Midi Kit (Art. Nr: 12145)
	QIAfilter Spin Maxiprep Kit (Art. Nr: 12263)

Table II.6 Restriction enzymes

Manufacturer	Enzyme	Buffer
Thermo Scientific	KpnI (Art. Nr: #FD0524)	FD Green Buffer (Art. Nr: #B72; Lot.133213) FD Buffer (Art. Nr: #B64; Lot.00151181)
	EagI (Art. Nr: #FD0334)	
	NheI (Art. Nr: #FD0974)	
	ApaI (Art. Nr: #FD1414)	
	ScaI (Art. Nr: #FD0434)	
	DpnI (Art. Nr: #FD1703)	
	BamHI (Art. Nr: #FD0055)	
	ScaI (Art. Nr: #FD0434)	
	PvuI (Art. Nr: #FD0624)	
	EcoRII (Art. Nr: #ER1921)	Orange Buffer (Art. Nr: #B05; Lot.00183458)
New England Biolabs	SacII (Art. Nr:#R3122)	Cut Smart Buffer (Art. Nr: B72045; Lot.0041305)
	NheI (Art. Nr:#R3131)	

Table II.7 Commercial plasmids

Origin	Plasmid ID
Addgene	p_101AATARP-CRE1916: pBS185 CMV-CRE
Promega	pGEM®-T Easy Vector (Art. Nr.: A1360: Lot 0059768)

III. Methods

1. Cell culture and bacterial strains

HeLa and McCoy cells lines (Table III.1) were cultured in growth medium at 37°C in a cell culture incubator under humidified atmosphere containing 5% CO_2. Before passaging, adherent cells were washed once with PBS (Invitrogen) and 1 ml of Trypsin (0.05%)-EDTA, trypsin as a protease to hydrolase proteins for cell dissociation and EDTA used as a chelating agent that binds to calcium and prevents clumping of cells, were added per 75 cm3 culture flask and cells were incubated approximately 3 min at 37°C until they detached. Cells were resuspended in growth medium and transferred into fresh culture flasks in a 1:4 dilution. Cells were maintained in culture and split every two or three days to avoid complete confluence.

Table III.1 Cell lines used

Cell Line	Cultured in
HeLa229 *Homo sapiens*, cervix, epithelial (ATCC CCL-2.1)	RPMI 1640 medium with 2 mM L-glutamine and 25 mM HEPES (Invitrogen), 10% FCS, heat-inactivated (Biochrome).
HeLa229_mT/mG *Homo sapiens*, cervix, epithelial (Toelle *et al*., unpublished)	RPMI 1640 medium with 2 mM L-glutamine and 25 mM HEPES (Invitrogen), 10% FCS, heat-inactivated (Biochrome).
McCoy cells *Mus musculus*, fibroblast (ATCC CRL-1696)	DMEM medium with 10% FCS, 25 mM HEPES (Invitrogen), 1 mM Na-Pyruvate, 2 mM L-glutamine and 10% FCS, heat-inactivated (Biochrome).

HeLa229_mT/mG (reporter cell line) is an in-house constructed from HeLa cell line, based on the cell line described by Muzumdar and colleagues (2007). They were transduced with a construct (pLenti-mTmG – 12807bp) under CAG promoter, making the cells susceptible to a CRE recombinase-induced switching from red fluorescence to green fluorescence. Afterwards, these cells were selected for puromycin resistance and sorted via FACS for stable expression of membrane tagged tdTomato (mT) (unpublished).

Four different bacterial strains were used in this work, (Table III.2 and Table III.3) with different applications. *Chlamydia trachomatis* serovar D and L2 were used for infection of the cell lines described in Table III.1 and for transformation. *Ctr* L2 elementary bodies (EBs) were purified from infected HeLa229 cells. Two *E. coli* strains were used as competent cells for achievement of clones with the constructed plasmids (Table III.3).

Table III.2 Bacterial strains used for infection and transformation

Strain	Origin
Chlamydia trachomatis Serovar D	Human cervix (UW-3/Cx ATCC VR-885)
Chlamydia trachomatis Serovar L2	Lymphatic isolate (L2/434/Bu ATCC VR-902B)

Table III.3 Bacterial strains used for the cloning

Strain	Genotype	Origin
Escherichia coli TOP10F‘	F'[lacIqTn10(tetR)]mcrAΔ(mrr-hdRMS-mcrBC)φ80lacZΔM15ΔlacX74deoRnupGrecA1araD139 Δ(ara-leu)7697galUgalKrpsL(StrR)endA1λ-	Invitrogen (pHuLUC3/TOP10 ATCC PTA-10989)
Escherichia coli JM110	rpsL (Strr)thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44∆(lac-proAB) [FtraD36proABlacIqZ∆M15]	Stratagene (pBMS2000-PPFOH-PDHmod ATCC PTA-4520)

2. Software for data analysis

During this work several software packages were used for different purposes: Clone Manager 9 to plan the cloning; Image J, Photoshop CS5, VisiView, ScanR (Olympus) for acquirement and treatment of photos and images; CorelDRAWX4 for the design of schemes, Prism5 and Microsoft Excel for data analysis.

3. Bacterial DNA extraction

Chlamydia trachomatis DNA was extracted using the phenol/chloroform method. Gram negative bacteria ($1x10^8$ CFU) were resuspended in lysis buffer and then incubate 1 h at 37°C. Phenol/chloroform/isoamyl alcohol (1:1) was added and the phases were mixed by repeatedly inversion of the tube. The sample was spun-down at maximum speed and a white layer (protein layer) is visible at the aqueous:phenol/chloroform interface. The upper aqueous phase was transferred carefully to a new tube. To remove the phenol, an equal volume of chloroform was added to the aqueous layer and the sample was spun-down again at maximum speed for 5min. The aqueous layer was transferred to a new tube. To precipitate the DNA, ETOH was added and mixed gently. After 30 min Incubation at -20°C for the sample and spun down at maximum speed for 15 min at 4°C. The supernatant was discarded and the DNA pellet

was rinsed with 70% ethanol and spun-at maximum speed. The supernatant was carefully discarded, the DNA pellet was air-dried and resuspended in TE buffer.

4. Chlamydia trachomatis infection

HeLa or McCoy cells (Table III.1) were infected, with different MOI's (1, 2, 5 and 10) of *Ctr* L2 and *Ctr* D, 24 h after the seeding in RPMI or in DMEM supplemented with 5% FCS (Infection Medium), and incubated for 2 h. 2 h.p.i. fresh medium was added and the infected cells were incubated for 2 d (in case of *Ctr* L2) or 3 d (in case of *Ctr* D).

5. Immunofluorescence staining

For fixation, permeabilization and blocking two methods were applied, depending on the antibodies used: (1) the infected cells were fixed and permeabilized with ice-cold methanol and incubated overnight at 4°C and later blocked with 0.5% BSA in PBS for 30 min at RT or (2) the infected cells were fixed with 3,7% paraformaldehyde for 10 min and permeabilized and blocked with 0.5% BSA-0.02% Tween-20/Triton X-100 in PBS for 30 min at RT. The primary antibody was added at the recommended dilution in blocking solution for 1 h at RT, followed by three washing steps with PBS. The secondary antibody was added at the recommended dilution together with Hoechst (1:1000 dilution) for host nuclei staining, diluted in blocking solution and incubated for 1 h in dark at room temperature followed by three washing steps with PBS. Finally PBS was added and the plates were analyzed by fluorescence or confocal microscopy.

6. Genetic transformation of Chlamydia trachomatis serovar D and L2

Plasmid DNA was prepared from a methylation deficient strain, E. coli JM110, *dam*$^{-}$*dcm*$^{-}$, strain using the Qiagen Plasmid MidiKit, as indicated by the manufacturer. The infected McCoy cells were cultured in DMEM supplemented with 10% heat inactivated FCS (Table III.1). The following protocol was adapted from procedures developed by Wang and colleagues (Wang *et al*., 2013a, 2013b, 2011); Agaisse and Derré (Agaisse and Derré, 2013); Song and colleagues (Song *et al*., 2013) and Bauler and Hackstadt (Bauler and Hackstadt, 2014).

For transformation of *Ctr* D and L2: a solution with 50 µl of stock 2.3x10^{9} *Chlamydia trachomatis* D EBs, 12 µg plasmid DNA and 200 µl $CaCl_2$ buffer was prepared and incubated for 30 min at room

temperature. Subsequently, antibiotic free medium was added and the bacteria added on McCoy monolayer in 6-well plates. The infected cells were centrifuged at 2400 rpm for 30 min (Thermo scientific Multifuge 3S-R Heraus) and allowed to settle at 35°C in 5%CO_2 for 2/3 days without cycloheximide or penicillin G treatment. 48 h.p.i. (for *Ctr* L2) or 72 h.p.i. (in case of *Ctr* D), the cells were detached using a 24 cm cell scarper (TPP #99002) and disrupted by vortexing using 3 mm glass beads (Roth, ArtA557.1 Lot.290159680) for 3 min. The cell debris were discarded by centrifuging 5 min at 1500 rpm (*Eppendorf* Centrifuge 5804R) and the cell suspensions were added to a new cell monolayer. In case of, *Ctr* serovar D the flasks were centrifuged at 2100 rpm for 15 min at 4º C (Beckman Coulter Allegra 6R Centrifuge), and allowed to settle. After 2 h, the medium was changed to new infection medium (DMEM medium with 5% heat-inactivated FCS, 25mM HEPES (Invitrogen), 1 mM Na-Pyruvate, 2 mM L) with penicillin G selection and 1 µg/ml of cycloheximide (Sigma) for McCoy cells and/or for HeLa cells as well (RPMI 1640 medium with 2 mM L-glutamine and 25 mM HEPES (Invitrogen), 5% heat inactivated FCS) with penicillin G selection and 1 µg/ml of cycloheximide (Sigma). At Passage 4, one T25 cm^3 flask lysate from McCoy cells was added to a HeLa229 cell monolayer and vice-versa. The cultures were then selected with penicillin G during passages, as described in Table III.4 and Figure III.1.

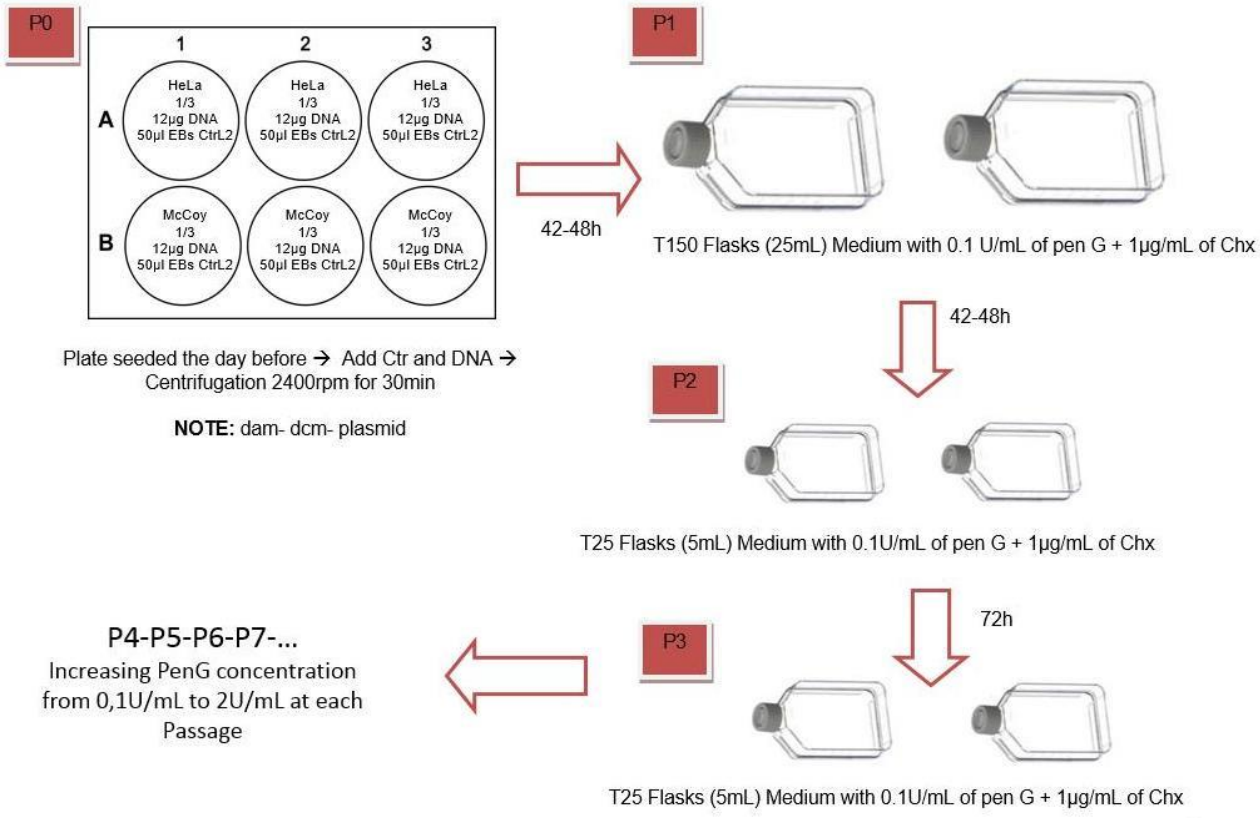

Figure III.1 Methodology adopted for *Chlamydia trachomatis* serovar L2 (*Ctr* L2) genetic transformation. In the case of *Ctr* D, the days were adapted to its life cycle as shown in Table III.4.

Table III.4 Transformation of *Chlamydia trachomatis* serovar D and L2. Information relative to every passage of transformation of *Chlamydia trachomatis* serovar D and L2.

Passage	Culture vessel	Penicillin G selection	Duration
Passage 1 (the content of the three wells is used)	T150 flask of McCoy	0.1 units/m (±0.006µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D
Passage 2 (use half of the T1 to each flask)	T25 flasks (one with HeLa and other with McCoy)	0.1 units/ml (±0.006µg penG)	3 d for *Ctr* L2 4 d for *Ctr* D
Passage 3 (use T2)	T25 flasks (one with HeLa and other with McCoy)	0.1 units/ml (±0.006µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D
Passage 4 (use T3)	T25 flasks (one with HeLa and other with McCoy)	0.1 units/ml (±0.006µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D
Passage 5 (use T4)	T25 flasks (one with HeLa and other with McCoy)	0.1 units/ml (±0.006µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D
Passage 6 (use T5)	T25 flasks (one with HeLa and other with McCoy)	1 units/ml (±0.06µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D
Passage 7 (use T6)	T25 flasks (one with HeLa and other with McCoy)	2 units/ml (±0.012µg penG)	2 d for *Ctr* L2 3 d for *Ctr* D

After Passage 7 the infection was propagated with increasing concentrations of penicillin G; for further preparation of transformed *Chlamydia trachomatis* stock.

7. Propagation and preparation of the chlamydial stock

For the preparation of *Ctr* stocks, *Ctr* were propagated in HeLa cells in T150 cm^3 cell culture flasks with 25 ml of growth medium. Cells were infected with lysates from earlier passages and incubated at 35°C in a cell culture incubator under humidified atmosphere with 5% CO_2 until time of harvest. 48 h.p.i., the cells were detached using a 24cm cell scarper (TPP #99002) and disrupted by vortexing using 3 mm glass beads (Roth, ArtA557.1 Lot.290159680) for 3 min. The cell suspensions were centrifuged at 1500 rpm at 4°C for 10 min (*Eppendorf* Centrifuge 5804R). The resulting supernatant was collected and centrifuged at 20000 rcf for 40 min at 4°C in a SS-34 rotor (Sorval RC 5C Plus) to pellet *Chlamydia* EBs. The harvested bacteria were resuspended in 5 ml of SPG buffer (4º C). A second centrifugation was performed and the pellet resuspended in 4 mL of SPG buffer (4º C). To dissolve any clumps, a G-23, a G-27 and lastly a G-30 gauge injection needles were used. *Chlamydia* suspensions were aliquoted and

frozen at -80°C. Before each experiment *Chlamydia* was freshly thawed. Presence of *Mycoplasma spp.* was excluded using the VenorGeM PCR assay.

To determine the infectivity of purified *Ctr* stock, serial dilutions of freshly thawed bacteria were prepared in infection medium wit 50 µl per well of each dilution inoculated into a 96F-well plate containing HeLa cells seeded one day before (5000 cells/well). 2 h.p.i. at 5% CO_2 and 37°C, the infection medium was replaced with 100 µl per well of fresh infection medium and incubated again under similar conditions. After 24 h, the cells were fixed, permeabilized and stained with the DAPI. The average number of inclusions per cell was determined for each dilution. The dilution of bacteria leading to the formation of one inclusion per cell upon infection was calculated and considered to have an MOI of 1.

8. Gene Cloning

Gene cloning is the use of a fragment of DNA from an organism where it naturally occurs, and putting it into a cloning host such as the bacterium *Escherichia coli*, even when subsequently it will be used to be transferred into another organism. It is necessary to clone the fragment of DNA into a proper cloning vector, with the right promoter that will be copied every time that the cell copies its own DNA. On this cloning vector, a plasmid, the fragment of DNA is inserted after cutting it using restriction enzymes and later ligation by DNA ligase. The ligation reaction efficiency can be increased by treating the cut vector in such a way that the two ends cannot be ligated to each other, but can still be ligated with an insert, by the treatment of the vector with alkaline phosphatase, removing the 5′ phosphates from the cut vector preventing it to ligate with itself (Lodge *et al.*, 2007).

A. DNA Manipulations, Plasmids and Primers

The main plasmids used and generated in this work and their main features are detailed in Table III.5 and the primers used are shown in Table III.6. Plasmids were constructed using standard DNA manipulations with proof-reading Phusion High Fidelity DNA polymerase (NEB #M053OS), restriction enzymes (Table II.6), T4 DNA Ligase (NEB and Promega), QIAquick PCR Purification Kit and Gel extraction kit (Qiagen), and the DNA extracted with the Plasmid Mini/Midi/Maxiprep kit (Qiagen and Thermo Scientific), according to the instructions of each manufacturer. *Ctr* genes, or parts of genes, were amplified by PCR from genomic DNA of strain L2/434 using custom oligonucleotide primers, Table III.6. The accuracy of the nucleotide sequence of all the inserts of the constructed plasmids was checked by DNA sequencing (MWG) (Supplementary Data 1).

Table III.5 Constructed plasmids

Plasmid ID	Genetic elements	Resistance	Amplified in
p2TK2-SW2_IncDProm-mCherry-IncDTerm	IncDProm-mCherry-IncDTerm IncD_Gene ID: 5859205	Ampicillin	*E. coli* JM110
pGEM®-T Easy Vector-TARP	TARP_GeneID:884231	Ampicillin	*E. coli* TOP10F'
p2TK2-SW2_IncDProm-IncDTerm	IncDProm-IncDTerm IncD_Gene ID: 5859205	Ampicillin	*E. coli* TOP10F'
p_101AATARP-CRE	IncDProm-IncDTerm IncD_Gene ID: 5859205 101AATARP_Gene ID:471472 CRE_Gene ID:10678	Ampicillin	*E. coli* JM110
p_TARP-CRE	IncDProm-IncDTerm IncD_Gene ID: 5859205 TARP_Gene ID:471472 CRE_Gene ID:10678	Ampicillin	*E. coli* JM110

Table III.6 Primers used

Primer	Name	Sequence
1	TARP from *Chlamydia trachomatis* L2 FW	CGGAGGAACCTTGGACAATA
2	TARP from *Chlamydia trachomatis* L2 RV	AGGGAGTGACCTGCTCTGAA
3	IncDPromoter FW (KpnI-Prom-NheI)	GTTTCGCCACCTCTGACTTG
4	IncDPromoter RV (KpnI-Prom-NheI)	TCATCCCGGGCCCTAGACGCTAGCACCTCACTTC GACAGATTTTAGC
5	IncDTerminator FW (ApaI-Term-EagI)	GTCTAGGGCCCGGGATGACATGTGATTCGCGTAG
6	IncDTerminator RV (ApaI-Term-EagI)	TTGAAGCGCTCCGGATAGTG
7	TARP FW (NheI-TARP-SacII-Linker)	GAGGCTAGCATGACGAATTCTATATCAGGTGATC
8	TARP 101AA RV (NheI-TARP-SacII-Linker)	AGGGTTGCTAGGGCTGGCCCGCGGTTTTCCAGCA ACGGCTTGGG
9	TARP full RV (NheI-TARP-SacII-Linker)	CATCCGCGGTCCTACGGTATCAATCAGTG
10	CRE FW (Linker-NaeI-CRE-ApaI)	GCCAGCCCTAGCAACCCTGGCGCCAGCAACGGCA GCGCCGGCATGCCCAAGAAGAAGAGGAAGG
11	CRE RV (Linker-NaeI-CRE-ApaI)	GAGGGGCCCTCGACCTAATCGCCATCTTCC

B. Cloning plan for p_101AATARP-CRE and p_TARP-CRE

The molecular cloning of p_101AATARP-CRE and p_TARP-CRE was designed first *in silico* using Clone Manager 9 software, the final cassette of each are presented in Figure III.2. The cloning was divided into three main steps in order to create two final plasmids (p_101AATARP-CRE and p_TARP-CRE), these two final plasmids will have the same backbone as the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid used (Table III.5) for establishing the protocol of transforming *Ctr* but instead of the mCherry protein, two recombinant TARP–CRE protein versions were inserted.

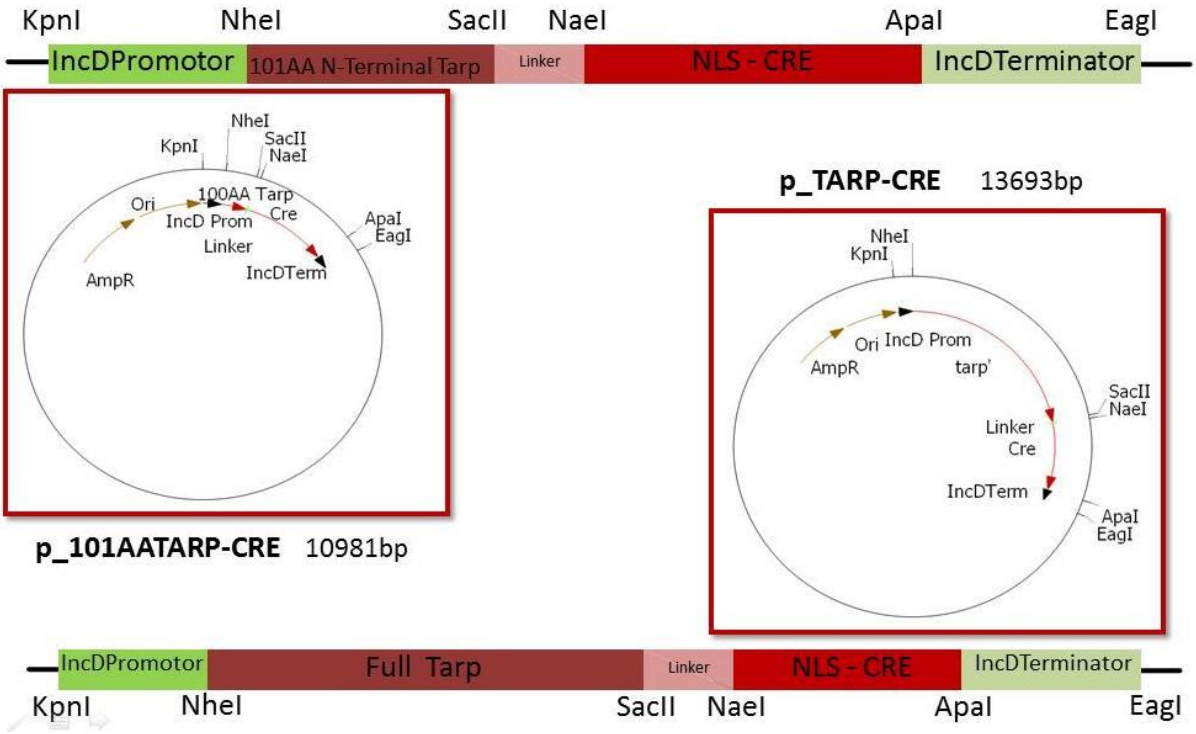

Figure III.2 Final plasmids constructs (p_101AATARP-CRE and p_TARP-CRE)

Part A: as summarized in Figure III.3, after the DNA extraction from *Chlamydia trachomatis*, TARP was amplified using primer 1 and 2 (Table III.6) and using the TA-cloning methodology (Promega Kit), TARP was inserted in pGEM®-T Easy Vector (Table III.5). From the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid (Table III.5) the IncDPromoter and IncDTerminator were amplified using primers 3-4 and 5-6 respectively. The first overlap PCR was performed using the products from the PCR of IncDPromoter and IncDTerminator as templates. The IncDpromoter-mCherry-IncDTerminator was cut from the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid using the restriction enzymes KpnI and EagI

and the product from the overlap PCR cloned inside creating the plasmid p2TK2-SW2_IncDProm-IncDTerm (Table III.5).

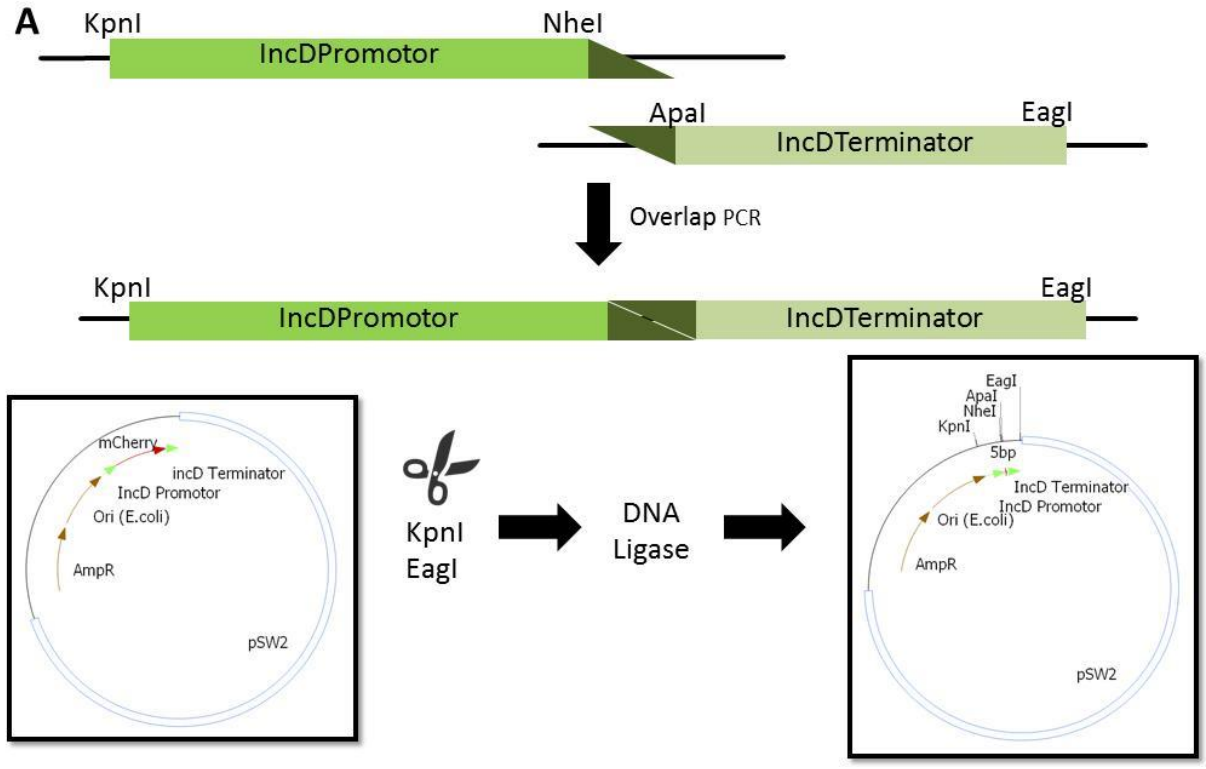

Figure III.3 Part A, resulting in the backbone plasmid p2TK2-SW2_IncDProm-IncDTerm.

Part B: as shown in Figure III.4, the 101AA TARP was amplified by PCR (reaction A) from pGEM®-T Easy Vector-TARP (Table III.5) using primer 7 and 8 (Table III.6). CRE was generated by PCR (reaction B) from the pBS185 CMV-CRE plasmid (Table III.5) using primer 10 and 11. An overlap PCR was performed using the matching products from reaction A and B are, in part, complementary between them, creating a product that was cloned inside of the plasmid p2TK2-SW2_IncDProm-IncDTerm (Table III.5) using the restriction enzymes ApaI and NheI.

Part C: finally to create p_TARP-CRE (Table III.5), as shown in Figure III.5, the full TARP was amplified by PCR from pGEM®-T Easy Vector-TARP (Table III.5) using Primer 7 and 9 (Table III.6) and the product was cloned into p_101AATARP-CRE using the restriction enzymes SacII and NheI (Table II.6).

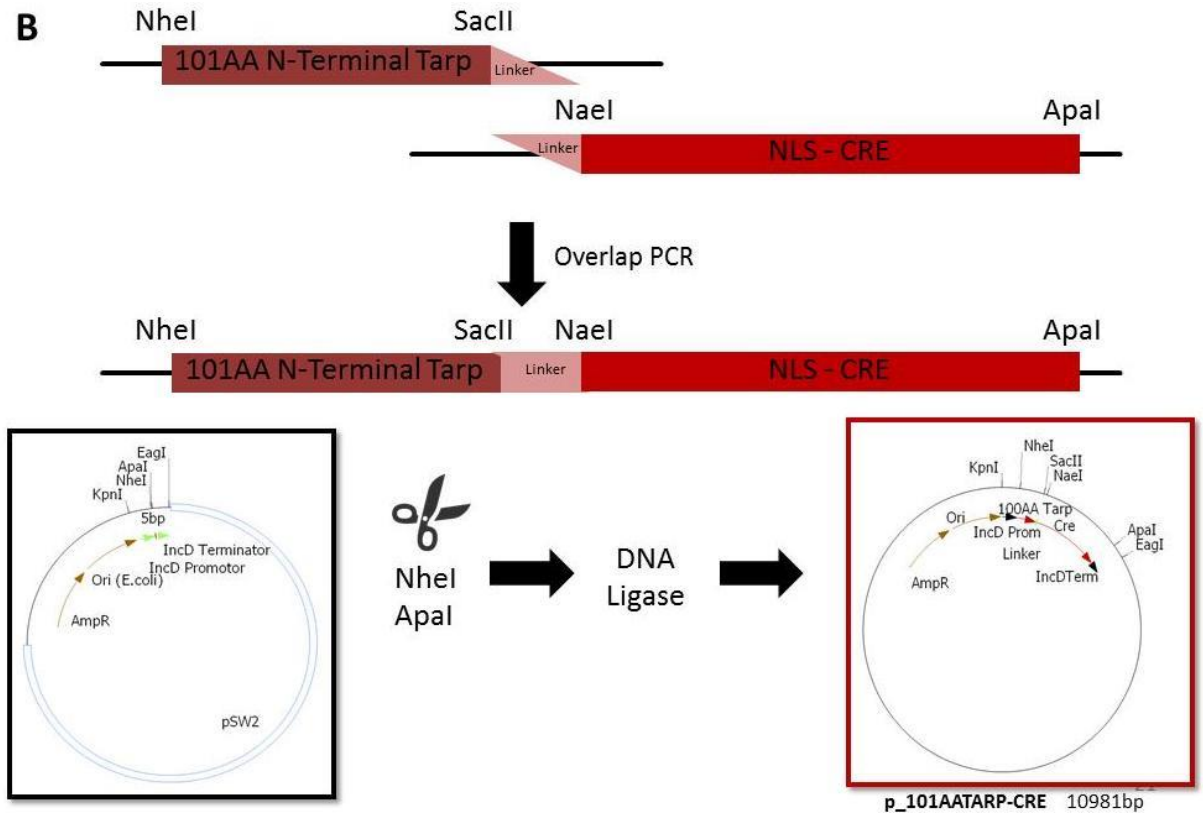

Figure III.4 Part B resulting in p_101AATARP-CRE.

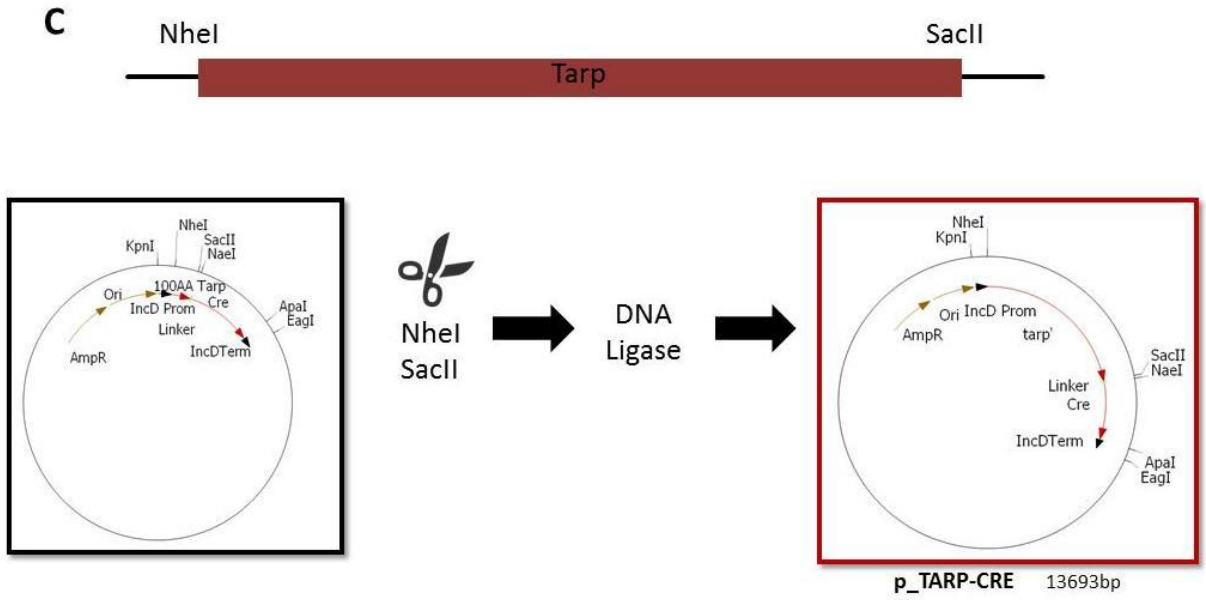

Figure III.5 Part C resulting in p_TARP-CRE.

The next methodology points will present a more detailed procedure for the cloning of p_101AATARP-CRE and p_TARP-CRE:

C. TA cloning

TA Cloning consists on the treatment of the fragment with a Taq polymerase in the presence of dATP, adding a single non template-directed deoxyadenosine (dATP) residue to the 3' end of the PCR product; the vector is then tailed with dideoxythymidine triphosphate (dTTP). Thusly, it is created a complementary one base pair sticky ends because a ddT-tailed vector can ligate directly to a PCR product that contains a 3'-overhanging dATP by forming a phosphodiester bond between the vector's 5'-P and the PCR product's 3'-OH group from the overhanging adenosine (Holton and Graham, 1991; Marchuk *et al*., 1991). The insert may be ligated to the T-vector in either direction; therefore TA cloning is ideal when both orientations of the insert DNA in the plasmid are required. This strategy is both simple and one hundred fold more efficient than blunt-ended ligation for the cloning of PCR products (Marchuk *et al*., 1991; Zhou and Gomez-sanchez, 2000).

In this study, TA Cloning was used to clone TARP from *Chlamydia trachomatis* extracted DNA into pGEM®-T Easy Vector for later use in the Cloning of p_101AATARP-CRE and p_TARP-CRE. The first step is the polyadenilation of the purified PCR fragment generated with Phusion DNA Polymerase in the presence of dATP (final concentration of 0.2 mM) and 10X NEB Reaction Buffer. The mixture was incubated at 70°C for 15–30 min. Subsequently, TARP was cloned into pGEM®-T Easy Vector (50ng) using 2x Rapid Ligation Buffer, pGEM®-T Easy Vector from Promega (50 ng), 16.6 ng (1/3) of the insert (adenilated product) and the T4 DNA ligase (3 Weiss unit/µl) from the Promega TA cloning Kit for 1 h incubation at room temperature.

The ligation product was used to transform *E. coli* TOP10F' competent cells like recommended for the manufacture using 50 µl of competent cells on a 2 mL tube, incubating it on ice for 30 min. The tubes were immersed in a 42°C water bath for 50sec. Immediately after, the cells were returned to ice for 2 min, adding later 750 µl of warm SOC medium to each sample. E. coli TOP10F' was incubated in a 37°C incubator/shaker, allowing the cells to recover and begin expressing penicillin resistance for 1 h (3 cycles of *E. coli* replication). After 1 h, 100 µl of each cells were seeded on IPTG-Xgal-Amp (0.1mM- 40ug/ml-100µg/ml) blue/white screening and antibiotic selection.

After an overnight incubation at 37º C, single blue and white colonies were observed and the white ones were selected based on the principle of α-complementation of the β-galactosidase gene (cells transformed with vectors containing recombinant DNA will produce white colonies and cells transformed with non-recombinant plasmids produce blue colonies). To select the right clone the DNA was extracted according to the protocol and followed by a restriction test.

D. Molecular Cloning - Standard Protocol

The initial inserts were generated by conventional PCR (Polymerase Chain Reaction) or overlapping PCR and then digested, as well as the vector used, with the same couple of restriction enzymes (Fast Digest from Thermo Scientific) for 1 h at 37º C. Inactivation of the restriction enzymes occurred at 65º C for 20 min. The insert was purified by the QIAquick PCR Purification Kit. After the first digestion the linearization of the backbone was checked by gel electrophoresis and later digested with the second enzyme in the same conditions. The double digested vector was then dephosphorylated using the FASTAP enzyme (Thermo Scientific #EF0654) as recommended by the manufactures. After the dephosphorylating the vector was ran in an electrophoresis gel without ethidium bromide (0.7% TAE Gel) and stained with methylene blue for further gel extraction of the double digested vector. Both vector and insert were quantified by the Nanodrop (peQlab Nanodrop spectrophotometer ND-1000) and by a gel electrophoresis using ImageJ for the calculation of the approximate quantity of DNA (pixel densitometry analysis), for further ligation. The ligation was performed overnight at 16º C using a 3:1, 1:3 and1:1 ratio of vector:insert ratio together with the 10x ligation buffer and 1 µl of T4 DNA Ligase from NEB. For ligation, it was always used two controls: (C1) Double-Digested Vector + Ligase and (C2) Double-Digested Vector without Ligase.

The transformation in E. coli TOP10F' or JM110 were performed as recommended by the manufactures and described above, using SOC medium and growing it overnight at 37º C in LB agar plates with the selective antibiotic (Ampicillin). The DNA was extracted, according to the protocol, for 6-12 selected single colonies and a digestion test was performed to check for presence or absence of the desired plasmid. The desired clone was submitted to a DNA extraction according to the protocol and the DNA stored at -20º C in TE Buffer.

E. Sequencing

Both constructed plasmids, p_101AATARP-CRE and p_TARP-CRE, were sequenced by MWG-eurofins, by the "SimpleSeq" service, using the Sanger sequencing method with a set of primers from 5´to 3´ spanning 400-500bp. The primers were designed in CloneManager9 and the samples sent according to the instructions required by the company. The data, provided by the company, was analyzed using CloneManger9.

9. Protein detection by Western Blot

For the sample preparation, 0.3 ml of an *E. coli* overnight culture with an optical density at 600 nm between 1.2 and 2.0; was centrifuge and the pellet resuspended in 60 µl of 2x Sample Buffer (Table II.2)

with DTT. The sample was boiled for 10 min at 95º C and vortex. The lysates were frozen at -20º C or loaded directly on the gel.

The SDS-PAGE was carried out using a BioRad (Mini Protean II^{TM}) system. A stock solution of 30% acrylamide plus 0.8% bisacrylamide was used to prepare a 8% polyacrylamide gel (stacking gel at 4% and resolving gel at 8%). For polymerization of the gel, 0.1% TEMED and 0.5% APS were added. 20µl of each sample and 8µl of pre-stained protein ladder (Thermo Scientific #SM1812) were loaded into the gel and proteins were separated at 120 V for 2 h.

Separated proteins by SDS-PAGE were transferred electrophoretically to Immobilon-P Polyvinylidene difluoride (PVDF) membranes using the wet blotting method (BioRad Mini Protean II^{TM} system). PVDF membrane was activated in methanol for 1 min and then rinsed in transfer buffer. Transferring protein was carried out for 2 h at 250 mA at 4°C.

Fot the immunoblotting, the membrane was blocked in solution containing 3% BSA in TBS-T for 30 min. Further, the membranes were incubated overnight at 4°C in blocking buffer containing primary antibody at the appropriate concentration (Table II.3). Membranes were washed with TBS-T twice for 20 min and incubated with a corresponding secondary antibody in blocking solution for 1 h at RT. After two washing steps with TBS-T, for 20 min each, developing reagents (Western Lightning Chemiluminescent Reagent Plus (1:1 Western LightningTM PLUS-ECL of Enhanced Luminol Reagent Plus (lot275-13061) and Oxidizing Reagent Plus (lot265-13061) – Thermo Scientific)) were applied to detect the specific binding of peroxidase. The developing was done in a with Amersha, HyperfilmTM ECL High performance chemiluminescence films – GE Healthcare Limited (28906837) with different exposure times.

IV. Results

1. *Establishment of the genetic transformation of Chlamydia trachomatis serovar D and L2*

To achieve the genetic transformation of *Ctr*, a set of published papers were studied (Agaisse and Derré, 2013; Bauler and Hackstadt, 2014; Wang *et al.*, 2013a, 2013b, 2011) in order to create a functional protocol for *Ctr* L2 and *Ctr* D transformation. For these transformations, a chlamydial plasmid-based shuttle vector described in 2013 was used (Agaisse and Derré, 2013).

A. Chlamydia trachomatis infection

In order to acquire practice and understand the chlamydial infection cycle, HeLa cells were infected with *Ctr* L2 and *Ctr* D, in a 12 well plate. A record (Figure IV.1 and Figure IV.2) of their phenotype was kept for six days. As shown, *Ctr* inclusions grew bigger in each observation until occupy the full area of the cell. After 4 days for *Ctr* L2 and 6 for *Ctr* D, most cells were lysed due to the infection.

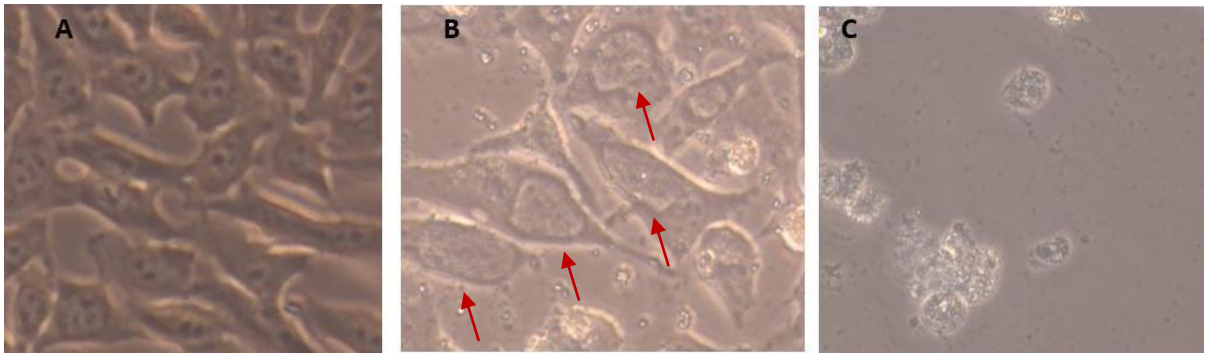

Figure IV.1 HeLa cells infected with *Ctr* L2. A- Non-infected HeLa cells; B- 100% HeLa cells infected with *Ctr L2* 2 d.p.i.; C- HeLa cells lysed by the infection with *Ctr* L2 4 d.p.i.. The infection medium was change 2 h.p.i. for synchronization of the infection. Red arrows show the chlamydial inclusions.

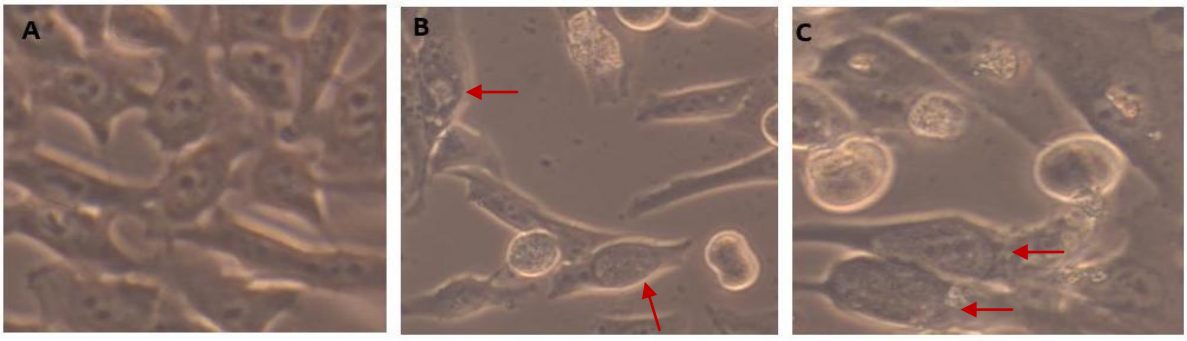

Figure IV.2 HeLa cells infected with *Ctr* D. A – Non-infected HeLa cells; B- HeLa cells infected with *Ctr* D 3 d.p.i.; C- 100% HeLa cells infected with *Ctr* D 4 d.p.i.. The infection medium was change 2 h.p.i. for synchronization of the infection. Red arrows show the chlamydial inclusions.

Regarding the lysing time, *Ctr* L2 lysed all the cells after 4 d.p.i. but in case of *Ctr* D that only happen 6 d.p.i. (data not shown), when the majority of what is visible were cell debris. This highlights that both serovars have different time cycles, with different consequences for the infected cells. For *Ctr* L2, 2 d (48 h) are enough to have full grown inclusions and consequently perform re-infection; however for *Ctr* D, 3 d (72 h) are necessary.

Four days post-infection (4 d.p.i.), some infected cells were fixed (Method 5) and stained for the presence of chlamydial inclusions (Cy3 – Red), cell actin (Cy2 – Green) and nuclei (Hoechst - DAPI) to monitor the infection by confocal microscopy, Figure IV.3. Is possible to see that on the non-Infected HeLa cells there were no inclusions, as expected but in the case of cell infected with *Ctr* L2, these ones have great number of inclusions, with a bigger number of inclusions on MOI5 than MOI1, as expected. For the cells infected with *Ctr* D, these have fewer inclusions than *Ctr* L2, although it is possible to see several ones on MOI1 but much more in MOI5 infections.

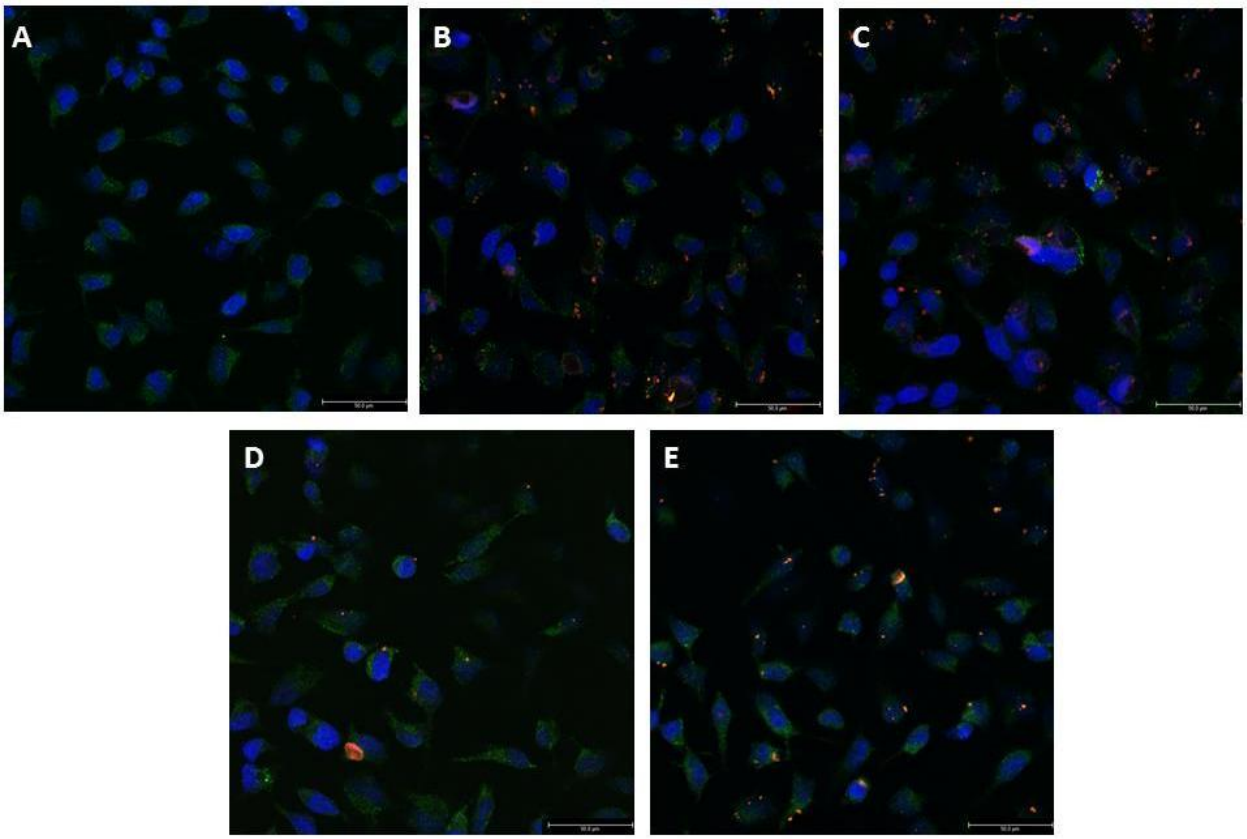

Figure IV.3 Immunofluorescence pictures of HeLa cells infected with *Ctr* L2 and D, 4 d.p.i.. Pictures acquired by confocal microscopy. A-Non-infected HeLa cells; B-Cells infected with *Ctr* L2 MOI1; C-Cells infected with *Ctr* L2 MOI5; D-Cells infected with *Ctr* D MOI1; E-Cells infected with *Ctr* D MOI5. Staining: Hoechst (blue) for DNA Cy2 (green) for actin and Cy3 (red) for chlamydial inclusions.

B. Plasmid shuttle vector - properties of p2TK2-SW2_IncDProm-mCherry-IncDTerm

p2TK2-SW2_IncDProm-mCherry-IncDTerm is a versatile shuttle plasmid (Table III.5 and Figure IV.4), generously provided by Agaisse and Derré. This plasmid was used for the transformation of *Ctr* L2 and *Ctr* D, allowing the expression of fluorescent proteins under the control of *Chlamydia trachomatis* IncD promoter and terminator (Agaisse and Derré, 2013). Since this shuttle plasmid will be used for transformation of *Ctr* L2 and *Ctr* D it was mandatory to demethylated it by amplification on a *dam dcm* negative *E. coli* strain (*E. coli* JM110). DpnI that only cleaves *dam* methylated DNA and EcoRII that does not cleave *dcm* methylated DNA. Thus, as shown in Figure IV.5, the shuttle plasmid, demethylated, was not digested by DpnI and it was digested by EcoRII creating 7 fragments, proving the demethylation of the plasmid. At the time of the amplification in *E. coli* TOP10F', the colonies and bacterial pellet observed were totally red, what drives the conclusion that the mCherry protein is being expressed and therefore concluding that IncD promoter works in *E. coli* as well, what was also described by Agaisse and Derré (2013).

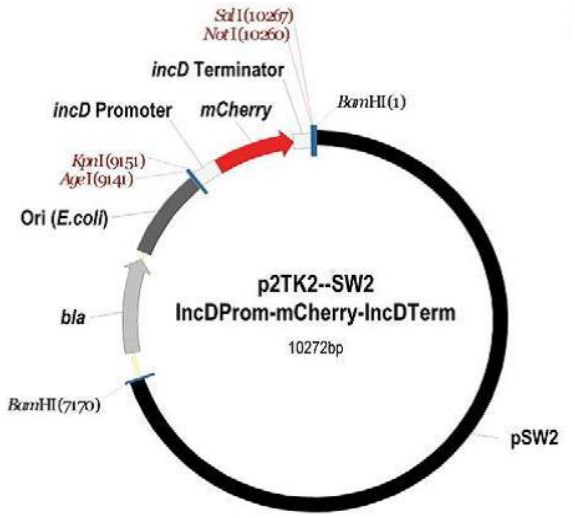

Figure IV.4 p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid (Adapted from Agaisse and Derré, 2013).

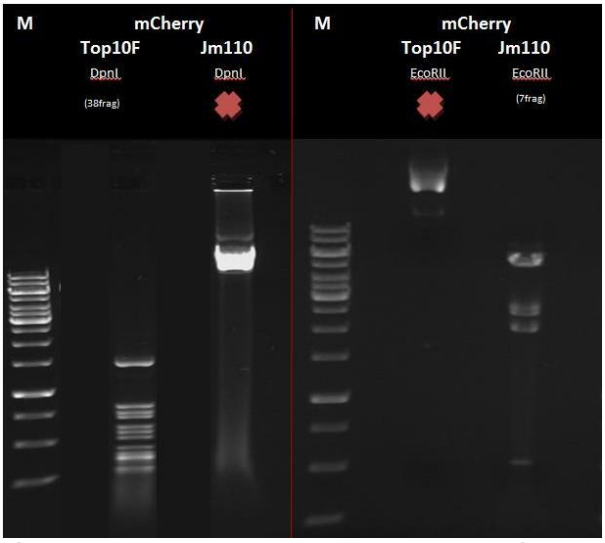

Figure IV.5 p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid. Plasmid digested with methylation sensitive enzymes, DpnI and EcoRII, for *dam* and *dcm* methylation sites, respectively, after amplification in *E. coli* JM110.

C. Transformation of Chlamydia trachomatis serovar L2 and D

The method for the genetic transformation of *Ctr* L2 and D, described in Methods (6), was applied to *Ctr* L2 and D. The different stages of both transformations, serovars L2 and D, are presented in Figure IV.6 and Figure IV.8, respectively, and a description of the observed phenotypes in Table IV.1.

Table IV.1 Description of the phenotype during *Ctr* genetic transformation

Passage	Comments
P1	Most inclusions were big and vacuolar (Lambden *et al.*, 2006; Skilton *et al.*, 2009).
P2	A few normal inclusions (non-vacuolar) were observed in this passage.
P3	Some normal inclusions were routinely recovered in this passage.
P4	Some normal inclusions were routinely recovered in this passage. The lysate from the HeLa flask was added in the one with McCoy cell monolayer and vice-versa.
P5 –P7	Normal inclusions were routinely recovered in this passage.
P8	Only normal inclusions were seen in this passage.

At Passage 0, without the addition of penicillin G, all inclusions presented a wild type phenotype, but at Passage 1, 2 days post-re-infection (2 d.p.r.i.), most of the inclusions were large and vacuolar demonstrating a persistence phenotype (Figure IV.6-A), as the ones already reported (Lambden *et al.*, 2006; Skilton *et al.*, 2009). At Passage 2, 3 d.p.r.i., a few normal inclusions were observed as well as some with the persistence phenotype (Figure IV.6-B-C). At Passage 4, some normal inclusions were observed (Figure IV.6-D)and after Passage 5 the number of these inclusions start to be higher (Figure IV.6-E-F), increasing at the same time the penicillin G (Figure IV.6-G-H). Nevertheless, for each experiment and passage, regular microscope observations of the infected cells allowed some flexibility in deciding precisely when the next passage should be done on the basis of sizes and phenotypes of the inclusions.

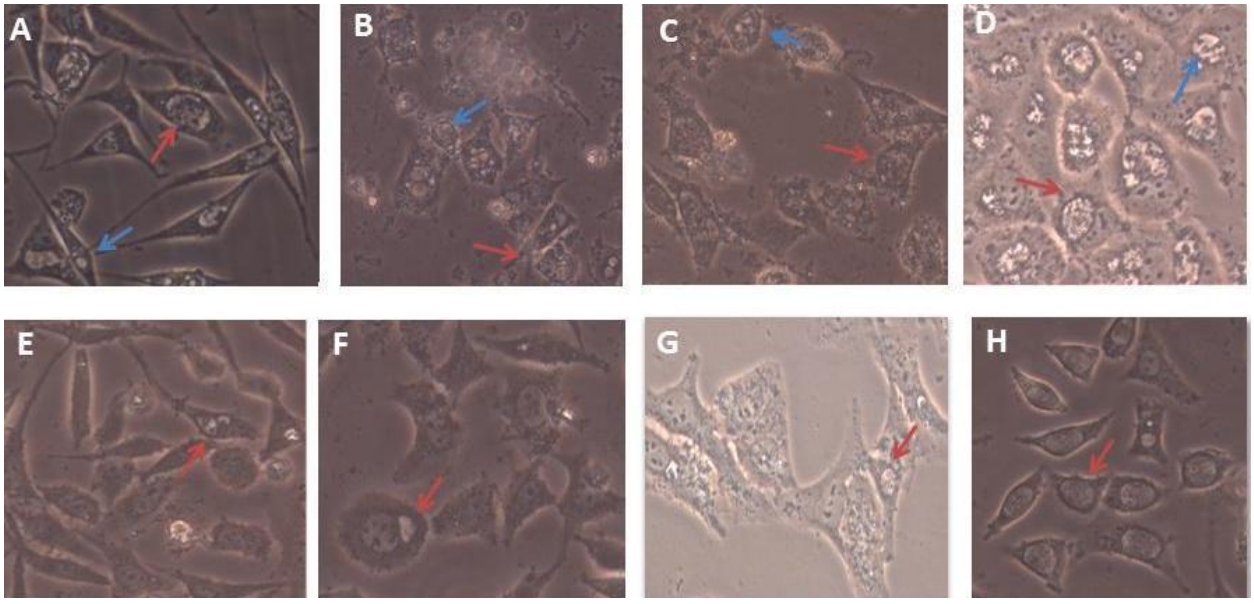

Figure IV.6 Genetic transformation of *Ctr* serovar L2 with p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid. A - P1 Day2; B - P2 Day2– 0.1 U/ml; C - P3 Day2; D - P4 Day1; E - P6 Day1; F - P6 Day2; G - P7 Day1; H - P8 Day2. Blue arrows show the vacuolar inclusions and red arrows the normal inclusions (transformed *Ctr*). From P1 to P5 0.1 U/ml penicillin G was used and after P6 1 U/ml.

To verify the transformation of *Ctr* L2 with the mCherry expression plasmid, on Passage 9 with 2 U/ml of penicillin G selection medium, live infected cells were observed under fluorescence microscope to visualize the red florescent signal of the transformed *Ctr* L2 inclusions; all the inclusions exhibited red fluorescence representing 100% of transformed *Ctr* L2 in the infection (Figure IV.7).

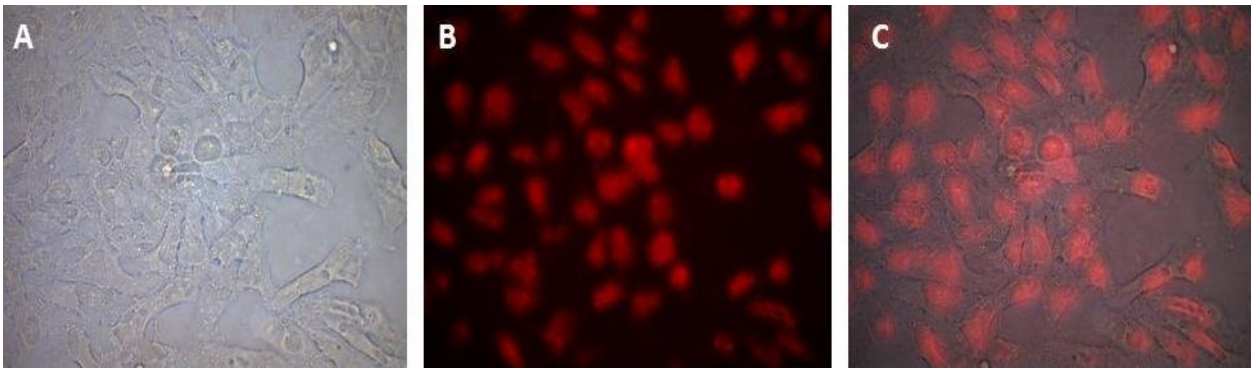

Figure IV.7 HeLa229 cells infected with transformed *Ctr* serovar L2. Acquired in a live cell microscope at Passage 9, 2 d.p.r.i., with 2 U/ml penicillin G selection. A-Brightfield; B-Cy3-inclusions; C-Overlay.

For the transformation of *Ctr* D, which has never been reported, we adopted a similar protocol. Normal inclusions were present during all passages under increasing selection of penicillin G selection (Figure IV.8).

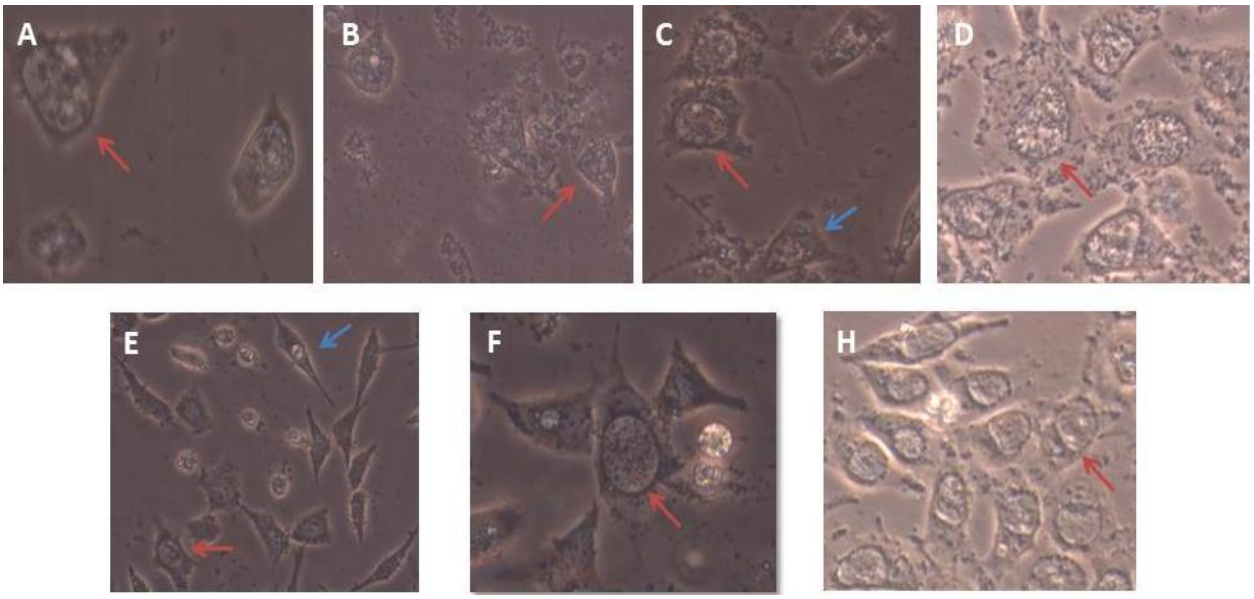

Figure IV.8 Genetic transformation of *Ctr* serovar D with p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid. A- P1 Day2; B- P2 Day2; C- P3 Day2; D- P4 Day1; E- P6 Day2; F-P8 Day2; G- P11 Day3. Blue arrows show the vacuolar inclusions and red arrows the normal inclusions (transformed *Ctr*). P1 to P5 0.1 U/ml penicillin G was used; from P6 to P10 1 U/ml and after P11 2 U/ml.

In the first passages the cells showed a persistence phenotype that gradually disappeared through passages due to the resistance acquired to penicillin G. In Figure IV.8-C and Figure IV.8-E is possible to see both phenotypes, persistence and WT for the transformed *Ctr*. The cells were then selected based on their resistance to penicillin G which is visible through a WT (Wild Type) phenotype of the inclusions. The tracking of size and phenotype of the inclusions during passaging of the infected cells allowed a decision of whether or not to proceed with the next passage.

Genetic transformation of *Ctr* D was verified as previously mentioned for *Ctr* L2 and, as observed in Figure IV.9, *Ctr* D presents red fluorescence for all the inclusions formed at passage 9, 3 d.p.r.i..

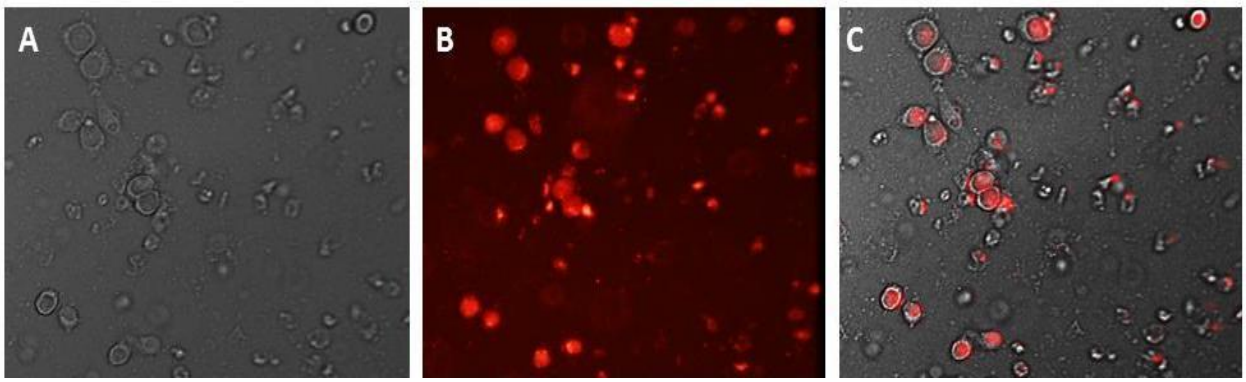

Figure IV.9 HeLa229 cell line infected with transformed *Ctr* D. Acquired in a live cell microscope at Passage 9, 2 d.p.r.i., with 2 U/ml penicillin G selection. A-Brightfield; B-Cy3-inclusions; C-Overlay.

D. Stock preparation

Subsequently to the transformation of *Ctr* L2 and *Ctr* D, both transformed serovars of *Chlamydia trachomatis* with p2TK2-SW2_IncDProm-mCherry-IncDTerm, were used for a stock preparation. After the preparation of the *Ctr* stocks, as described in Method 7, both serovars were used to infect HeLa cells. This experiment aimed to determine the titer, as well the ratio of transformed/not transformed *Ctr*. This ratio was not expected to be 100% in P0 (Passage 0) because the same was observed in a stock of transformed *Ctr* L2 with GFP. The infection was performed without penicillin G selection because its use in P0, right after the thawing, is stressful for the bacteria and when used in the stock all inclusions presented the persistence phenotype (data not shown).

For *Ctr* L2, titer of transformed *Ctr* of $8x10^6$ IFU, approximately 20-30% of the bacteria that are infecting HeLa cells were transformed in P0, as shown in Figure IV.10, through analysis by ScanR software. Nevertheless, when repassaging the stock with the majority of transformed *Ctr* L2, the population was re-established after only 3 Passages with 1U/mL of penicillin G selection (Figure IV.11).

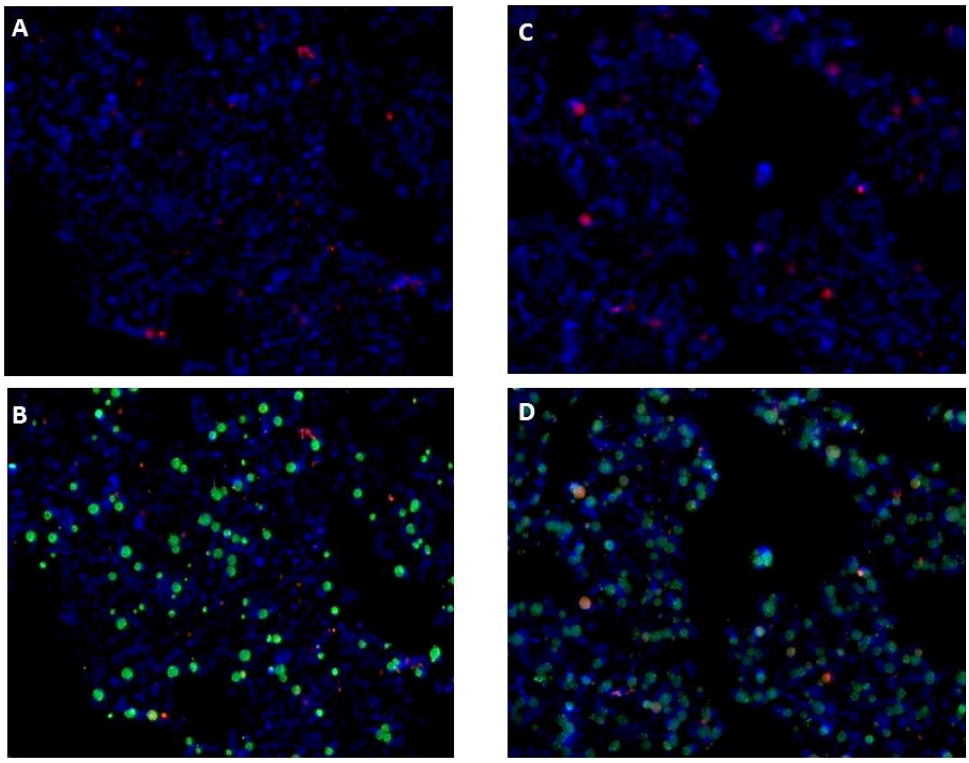

Figure IV.10 HeLa229 cells were infected with *Ctr* L2 stock. 2 d.p.i. the cells were fixed and labeled for *Chlamydia* inclusion (anti-MOMP) with Cy2 and Hoechst for nuclei using no penicillin G for selection. A- *Ctr* L2 mCherry in red dil1:4000; B- *Ctr* L2 mCherry and *Ctr* L2 WT in green dil1:4000; C- *Ctr* L2 mCherry in red dil1:1000; D- *Ctr* L2 mCherry and *Ctr* L2 WT in green dil1:1000.

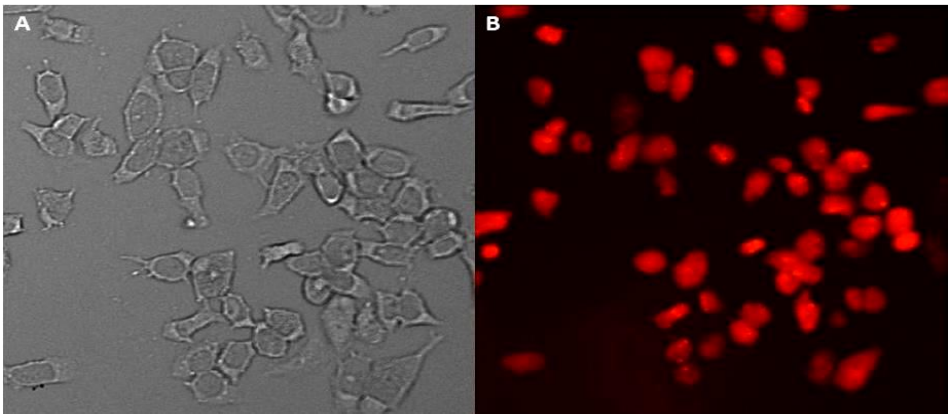

Figure IV.11 HeLa229 cells infected with *Ctr* L2 stock. Passage 3 after first infection with mCherry *Ctr* L2 stock, using 1U/mL penicillin G selection. A- Brightfield; B- Cy3 filter.

The inclusion size of the transformed *Ctr* L2 was also analyzed and studied by the ScanR software as presented in Figure IV.12. Compared to the WT *Ctr* L2, the sizes observed for the 20-30% transformed *Ctr* L2 at passage 0 (without penicillin G selection) and the non-transformed ones (70-80%) are approximately similar to the 24h.p.i. WT *Ctr* L2 inclusions.

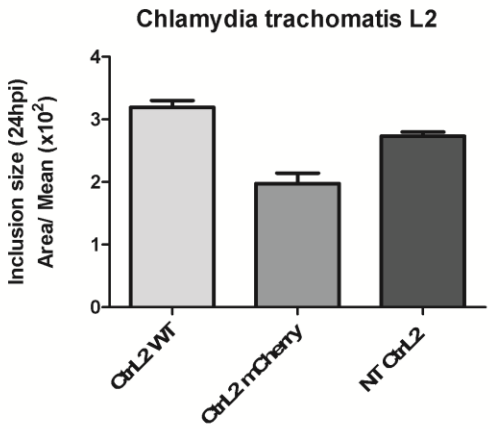

Figure IV.12 *Ctr* L2 inclusions size 24h.p.i.. *Ctr*L2 WT – *Chlamydia trachomatis* serovar L2 Wild type (Control); *Ctr*L2 mCherry – *Chlamydia trachomatis* serovar L2 transformed with the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid; NT *Ctr*L2 - *Chlamydia trachomatis* serovar L2 non-transformed present in the stock. Calculated by ScanR and presented using Prism5 software.

In case of the genetically transformed *Ctr* D stock, with the titer $9{,}7x10^7$ IFU, approximately 10-20% of *Ctr* D was transformed in P0 (without penicillin G selection), as shown in Figure IV.13. Although, when proceeding to re-infections with the cell lysates from this stock, most of the *Ctr* population was transformed at passage 3 using 1U/mL of penicillin G selection (Figure IV.14).

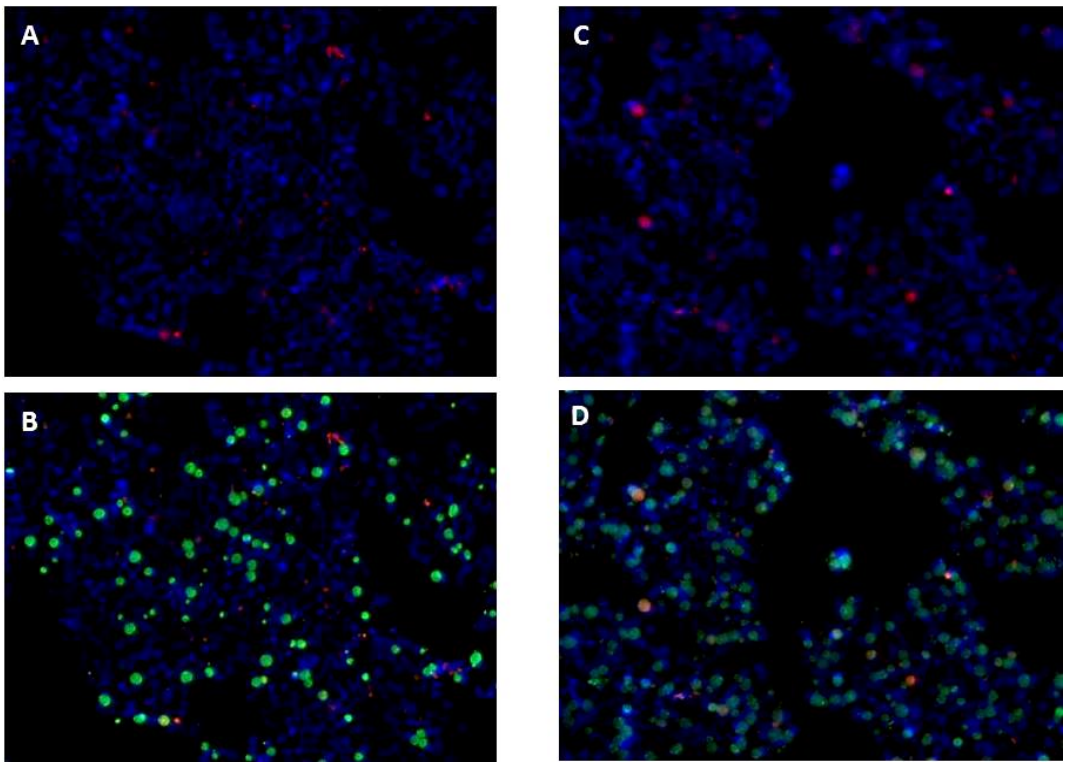

Figure IV.13 HeLa229 cell infected with the *Ctr* D stock. 2d.p.i. the cells were fixed and stained for *Chlamydia* inclusion (anti-MOMP) with Cy2 and Hoechst for the nuclei using no penicillin G for selection. A- *Ctr* D mCherry in red

dil1:4000; B- *Ctr* D mCherry and *Ctr* D WT in green dil1:4000; C- *Ctr* D mCherry in red dil1:1000; D- *Ctr* D mCherry and *Ctr* D WT in green dil1:1000.

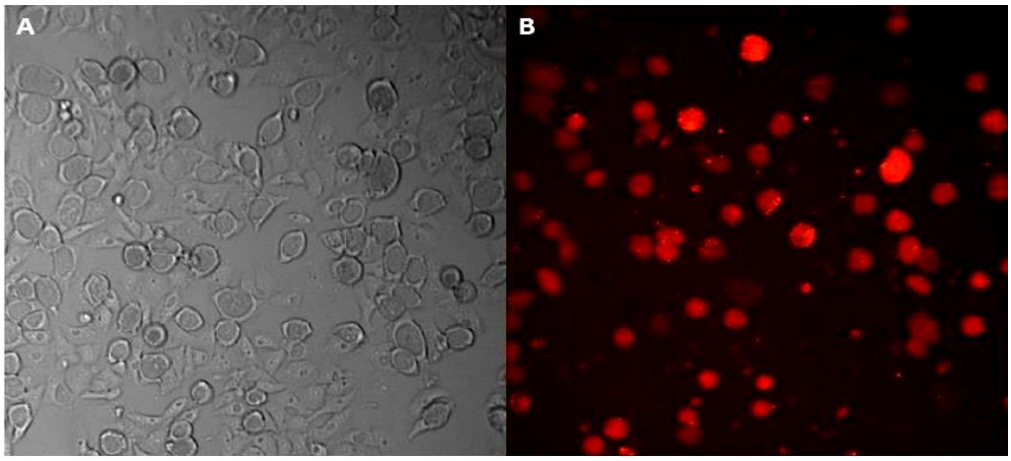

Figure IV.14 HeLa229 cell line infected with *Ctr* D stock. Passage 3 after first infection with *Ctr* D mCherry stock using 1U/mL penicillin G selection. A- Brightfield; B- Cy3 filter.

In relation to the inclusion sizes of the genetically transformed *Ctr* D, when compared to the WT *Ctr* D, the 10-20% transformed *Ctr* of the stock has approximately the same size for 24h.p.i. (Figure IV.15). However for the 80-90% non-transformed *Ctr* D presents in the stock, the inclusion size is bigger than expected when compared with the WT *Ctr* D.

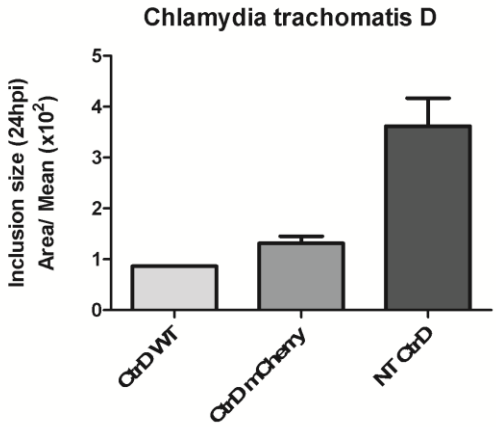

Figure IV.15 *Ctr* D inclusions size 24 h.p.i.. *Ctr* D WT – *Chlamydia trachomatis* serovar D Wild type (Control); *Ctr* D mCherry – *Chlamydia trachomatis* serovar D transformed with the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid; NT *Ctr* D - *Chlamydia trachomatis* serovar D non-transformed present in the stock. Calculated by ScanR and presented using Prism5 software.

2. Cloning of p_101AATARP-CRE and p_TARP-CRE

Briefly, the cloning started by the achievement of a plasmid with TARP, fusing it with the CRE recombinase in order to construct the p_101AATARP-CRE and p_TARP-CRE, described in Table III.5 and in Figure III.2.

A. Cloning TARP in pGEM®-T Easy Vector

As described in the methodology (3), bacterial DNA extraction was performed for both *Chlamydia trachomatis* serovars, D and L2, with a phenol:chloroform based protocol. The concentration achieved, given by the NanoDrop, was 12 ng/µl for *Ctr* D DNA and 20 ng/µl for *Ctr* L2 DNA.

The first step for the cloning of p_101AATARP-CRE and p_TARP-CRE was the cloning of *Ctr* TARP gene in pGEM®-T Easy Vector (Table III.5) via TA Cloning (Method (8.C), since it is an easy and fast way to clone an insert into a cloning vector in a stable manner. Initially, the TARP gene was amplified by PCR, from the previously extracted DNA of *Ctr* L2 and *Ctr* D, using primer 1 and primer 2 (Table III.6), the products generated (4kb) are shown in Figure IV.16.

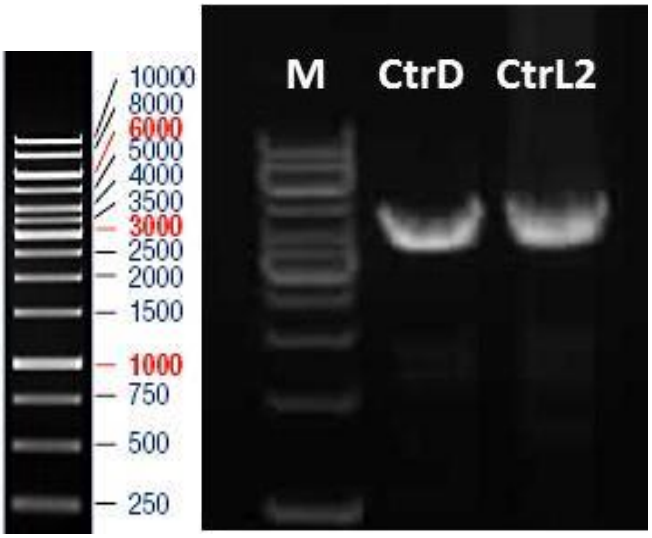

Figure IV.16 TARP gene (approximately 4kb) in pGEM®-T Easy Vector from *Ctr* L2 and *Ctr* D. Amplified with Primer 1 and Primer 2 (Table III.6). M-1kb Marker (Fermentas); CtrD-TARP gene from Ctr serovar D; Ctr2-TARP gene from Ctr serovar L2.

Subsequently, both fragments (TARP from *Ctr* L2 and *Ctr* D) were polyadenylated and ligated overnight with the pGEM®-T Easy Vector. The Figure IV.17 shows ScaI digestion profile. The expected sizes are 2366bp and 4340bp as it is possible to observe for clones D4, D5 and D7 for Ctr D and L1, L3 and L8 for Ctr L2. The samples selected for the subsequent steps were D4, D7 from Ctr D and L3 and L8 from Ctr L2.

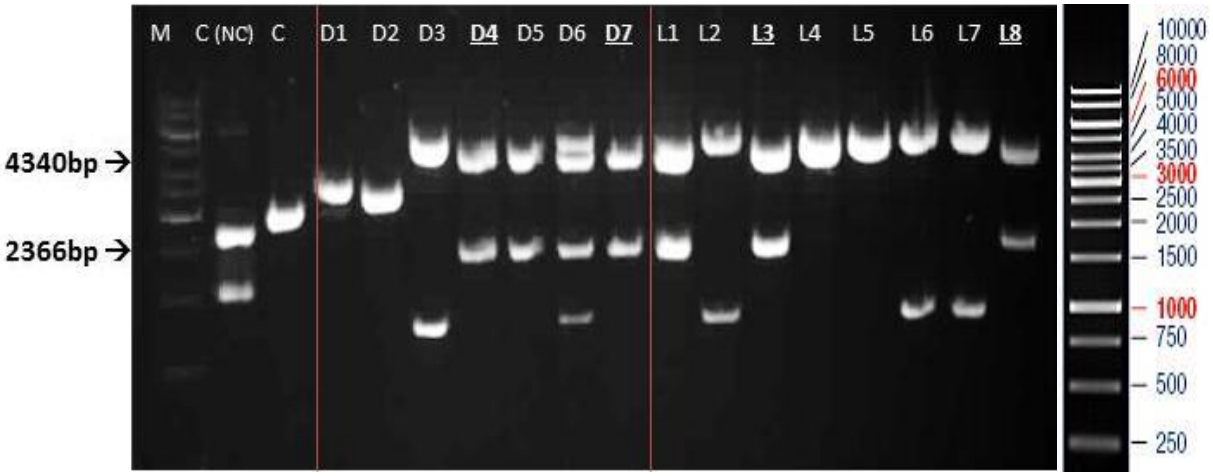

Figure IV.17 TARP in the pGEM®-T Easy Vector digested with ScaI. Digestion made to check the clones generated by transformation in *E. coli* TOP10F'. M-1kb Marker (Fermentas); C(NC) – control plasmid not digested; C-Control plasmid digested with ScaI.

B. Backbone plasmid construct (p2TK2-SW2_IncDProm-IncDTerm)

In order to construct p_101AATARP-CRE and p_TARP-CRE it was necessary to create a backbone plasmid that already possesses an antibiotic resistance (ampicillin), an origin of replication in *E. coli* and a *Ctr* promoter and terminator. The chosen plasmid was the p2TK2-SW2_IncDProm-mCherry-IncDTerm, used on the genetic transformation of *Ctr* L2 and D described above (Agaisse and Derré, 2013).

In order to use this plasmid it was necessary to remove the mCherry sequence to later substitute it with TARP-CRE recombinase fusion protein sequences for the construction of p_101AATARP-CRE and p_TARP-CRE. In order to overcome the absence of restriction sites confining the mCherry sequence, IncDPromoter and IncDTerminator were amplified with primer 3 and primer 4 (Figure IV.18-A) and primer 5 and primer 6 (Figure IV.18-B), respectively. After PCR purification of both products, an overlap PCR was performed with the products described. Both fragments were complementary to the each other; this complementary was given by the IncDPromoter RV primer and IncDTerminator FW primer (Figure IV.18-D). The product generated by the overlap PCR was 674bp length (Figure IV.18-C) and was then extracted by PCR Gel extraction kit from a 0.7% TAE electrophoresis gel stained with methylene blue.

The overlap PCR product (Figure IV.18-C) was digested with KpnI and EagI as well as the p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid to perform ligation. The ligation product was used to transform *E. coli* TOP10F' from which ten clones were analyzed by restriction digestion. Figure IV.19, shows the profile of 10 clones double digested with KpnI and EagI. Clones 6, 8 and 10 have created the expected fragments (9164bp and 414bp) for the required plasmid (Figure IV.20).

As this was the first cloning step the transformation it did have a big efficiency and it was not necessary to perform a big screening to find the desirable clone, with plasmid p2TK2-SW2_IncDProm-IncDTerm.

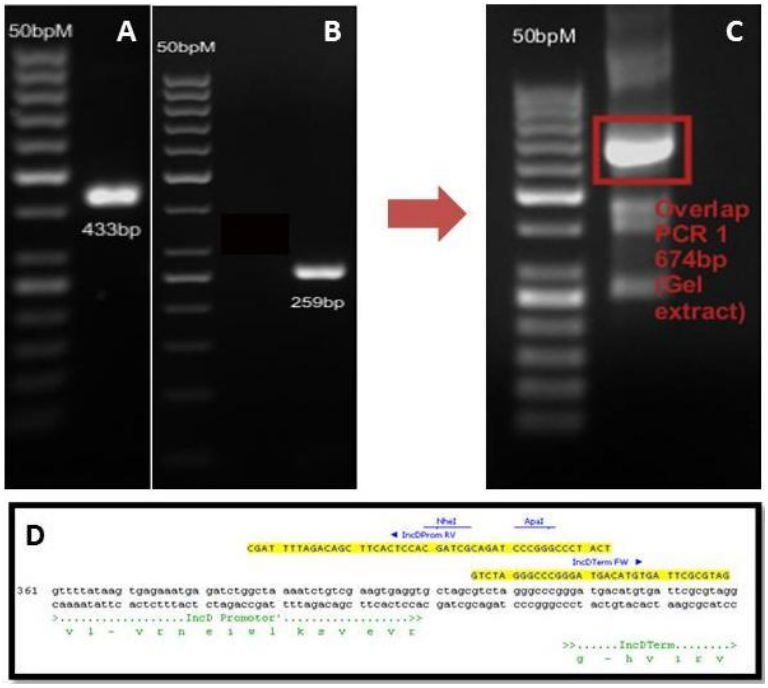

Figure IV.18 Overlap PCR of IncDpromoter and IncDTerminator. A- IncDPromoter amplification; B- IncDTerminator amplification; C- Product of the overlap PCR; D- Graphic scheme of the overlapping region necessary to perform the overlap PCR, generated in Clone Manager 9.

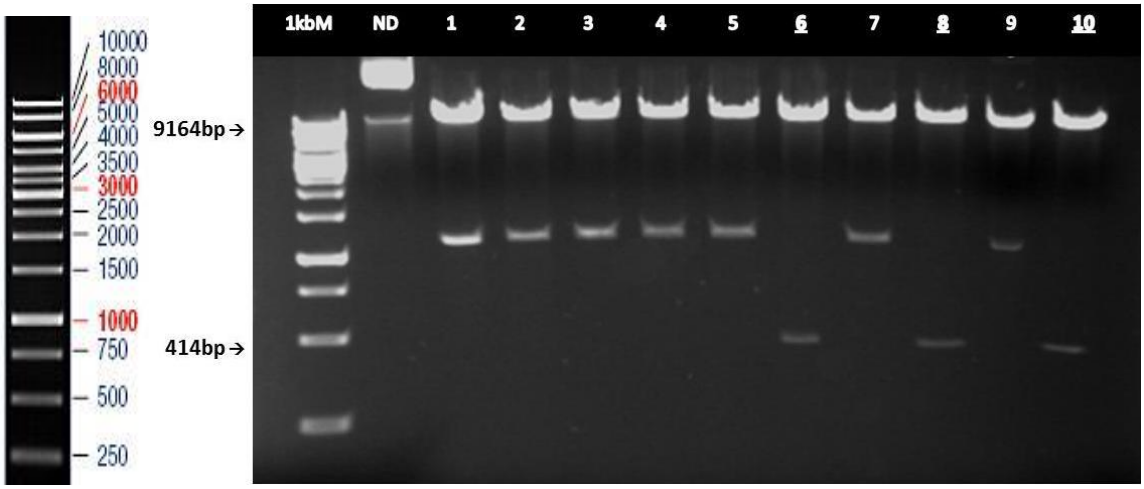

Figure IV.19 Restriction test for 10 *E. coli* clones transformed with the Ligation product using KpnI and EagI enzymes. 1kbM- 1kb DNA Marker.

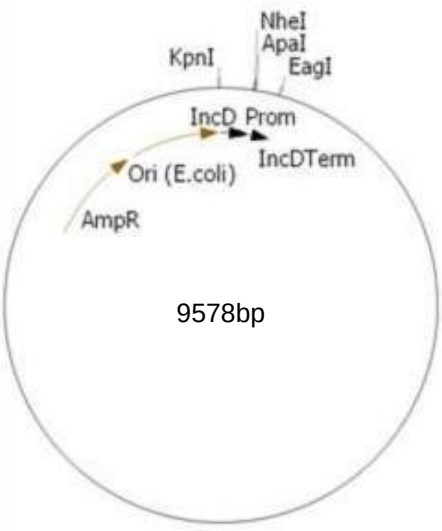

Figure IV.20 Backbone p2TK2-SW2_IncDProm-IncDTerm plasmid construct. Generated in Clone Manager 9.

C. p_101AATARP-CRE construction

To generate p_101AATARP-CRE , 101AA TARP and CRE were amplified with primers 8 and 9 (336bp product) and primers 10 and 11 (1109bp product), respectively, as shown in Figure IV.21-A and Figure IV.21-B. Samples were purified and an overlap PCR was made (Figure IV.21-C) due to the complementary given by the primers 9 and 10 in the initial PCRs (Figure IV.21-D).

The backbone plasmid created before (p2TK2-SW2_IncDProm-mCherry-IncDTerm) was digested with NheI restriction ezyme first and after with and ApaI restriction enzyme, as shown in Figure IV.22. The insert generated with the overlap PCR was run on a gel and purified. Insert and vector weredigested with NheI and ApaI, as it is showed in Figure IV.23. Densitometric analysis (performed with ImageJ) were performed to quantify the samples. Insert and vector were ligated overnight.

The ligation product was transformed in *E. coli* Top10F´ obtaining numerous colonies. 40 colonies were screened by PCR using the Primers 7 and 11 (Table III.6). The results from the PCR were analyzed in an electrophoresis gel, and, as shown in Figure IV.24, Clone number 40 is the only one showing the correct 1427bp band.

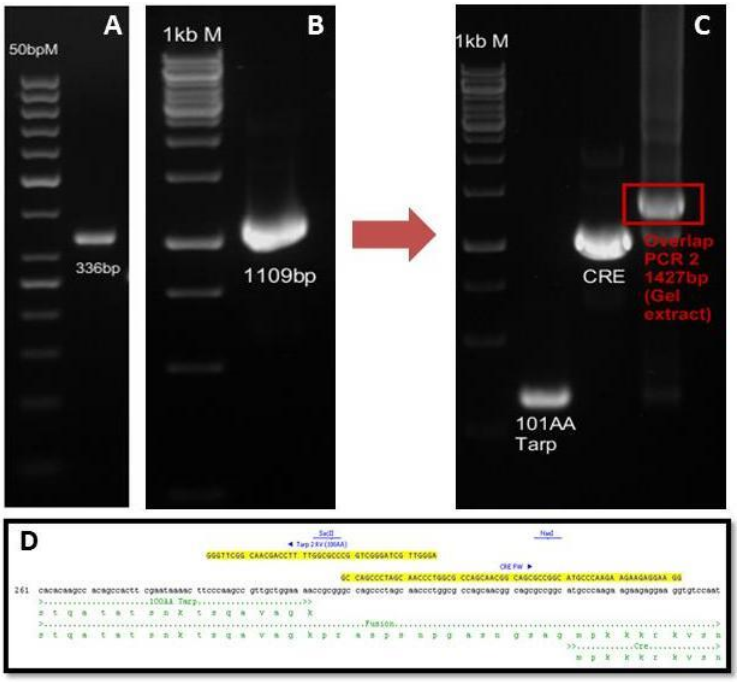

Figure IV.21 Overlap PCR of 101AA N-Terminal TARP and CRE. A- 101AA N-Terminal TARP amplification; B- CRE amplification; C- Product of the overlap PCR; D- graphic scheme of the overlapping region necessary to perform the overlap PCR, generated by Clone Manager 9.

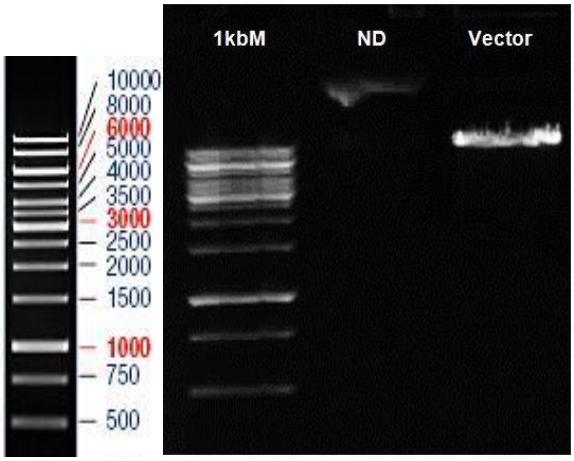

Figure IV.22 Gel to check linearization of the p2TK2-SW2_IncDProm-IncDTerm plasmid with NheI (9577bp).

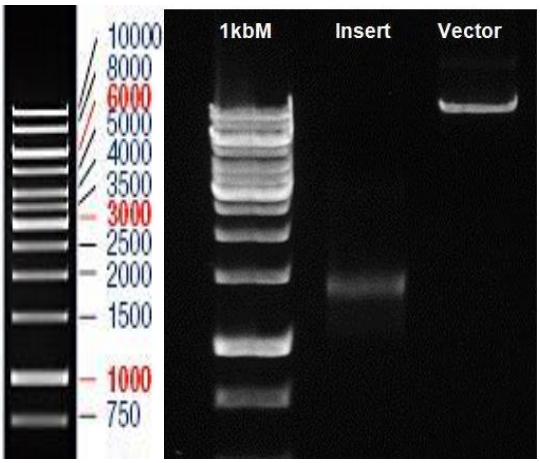

Figure IV.23 Gel Quantification of the vector (9562bp) and insert (1419bp) used for the cloning.

Subsequently, Clone 40 was tested with restriction enzymes KpnI, EagI, ApaI and NheI (Figure IV.25) for further confirmation for presence of p_101AATARP-CRE (Figure IV.26). The sequenced of the region of the insert in this plasmid (101AATARP-CRE) is available in Supplementary Data 1.A.

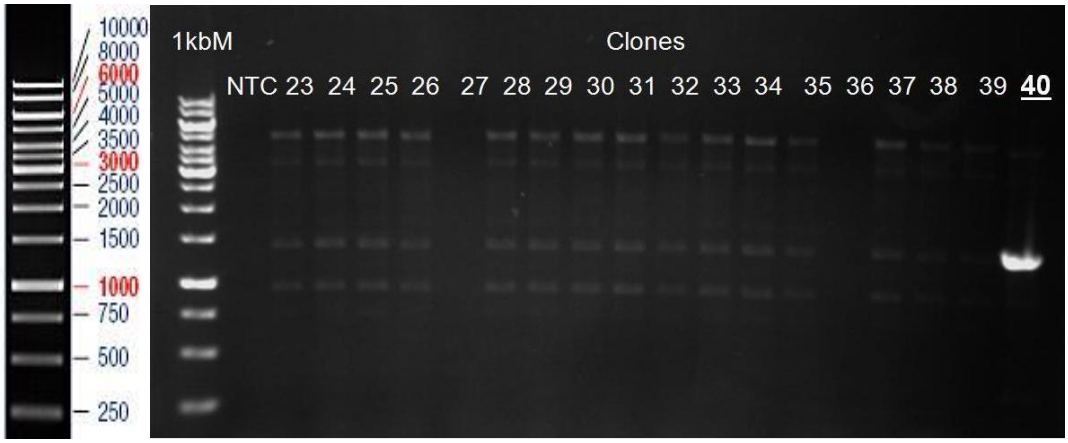

Figure IV.24 Colony PCR of 18 of the 40 E. coli clones. Analyzed using Primer 3 and 6 from table, creating an expected product of 1427bp. NTC- Non-Template Control.

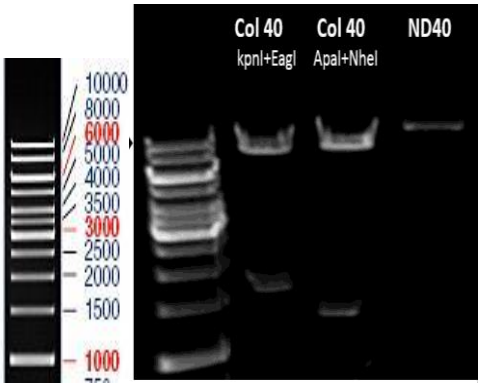

Figure IV.25 Diagnostic restriction digest of Clone 40. Restriction with KpnI and EagI creating two bands of 9163bp and 1818bp and ApaI and NheI creating two bands of 9562bp and 1419bp, as expected.

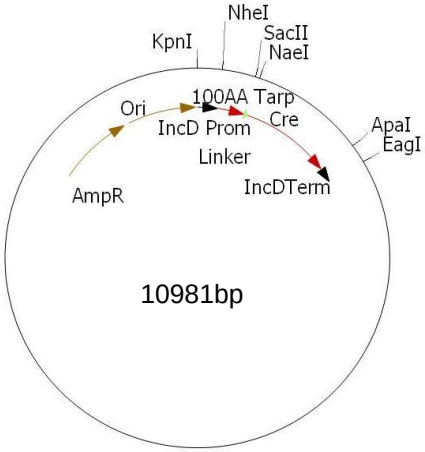

Figure IV.26 p_101AATARP-CRE construct (10981bp). Generated in Clone Manager 9.

D. p_TARP-CRE construction

In order to generate p_TARP-CRE, TARP was amplified by PCR from pGEM®-T Easy Vector-TARP (Table III.5) and digested with SacII and NheI. p_101AATARP-CRE was subsequentially digested with the same enzymes, first with SacII (Figure IV.27) and after with NheI (Figure IV.28). This plasmid was dephosphorylated and checked on a gel (Figure IV.29).

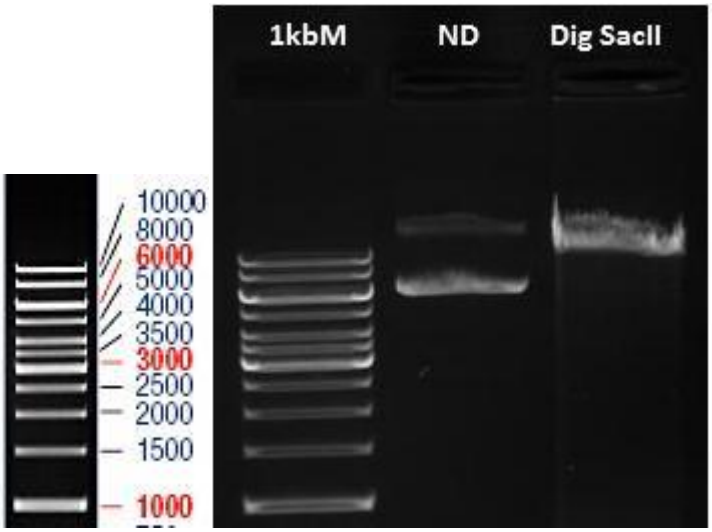

Figure IV.27 Gel to check linearization of the p_101AATARP-CRE plasmid with SacII. 1kb Marker from Fermentas was used. ND – Non-digested plasmid; Dig SacII – p_101AATARP-CRE digested with SacII.

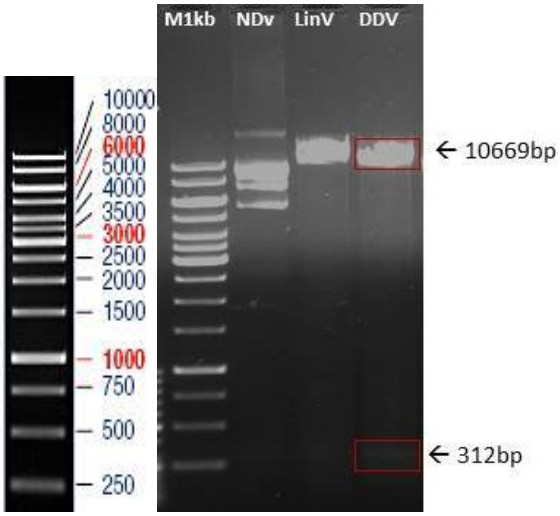

Figure IV.28 Double digested vector by NheI and SacII. M1kb – 1kb Marker (Fermentas); NDv- Non-digested vector; LinV – Linearized vector; DDV- Double-digested Vector dephosphorylated (creating a band of 10669bp and 312bp).

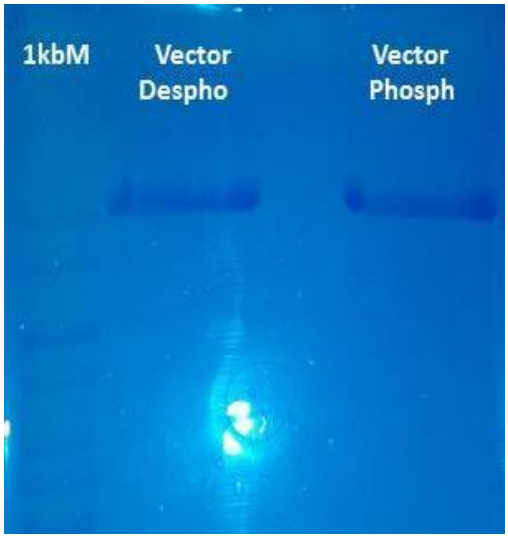

Figure IV.29 0.7% TAE Gel stained with methylene blue. This dephosphorylated vector was used for posterior gel extraction. 1kbM – 1kb Marker (Fermentas).

Samples were quantified with densitometric analysis (Figure IV.30) to calculate the ratios (3:1) for the ligation reaction.

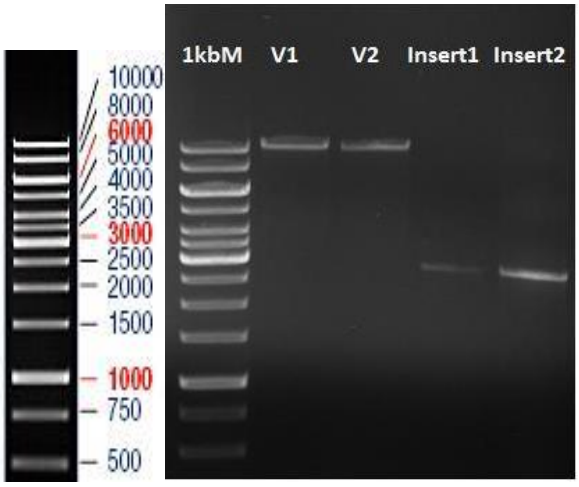

Figure IV.30 Gel quantification of vector (10669bp) and insert (3024bp) for p_TARP-CRE. Two samples of the Double-digested Vector (V1 and V2) and two samples of the Full TARP Insert (Insert1 and Insert2). 1kbM - 1 kb Marker (Fermentas).

E. coli TOP10F' was transformed with the ligation products, from which 14 colonies were analyzed with the restriction enzyme PvuI, which should create two bands (8601bp and 5092bp), as created by clone 8 in Figure IV.31.

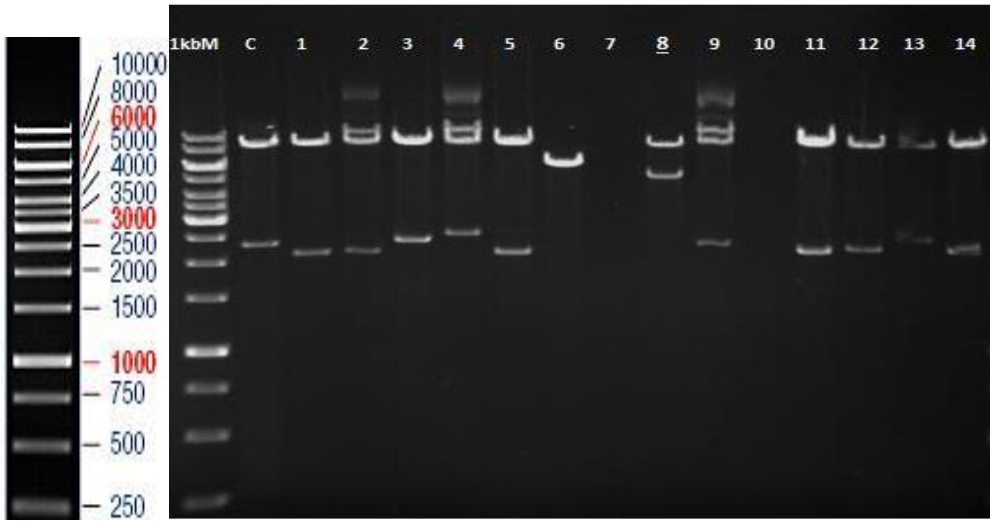

Figure IV.31 Restriction test using PvuI enzyme for 14 E. coli clones. The Control is the p_101AATARP-CRE digested with PvuI creating a fragment of 8601bp and 5092bp- 1kbM – 1kb DNA Marker.

Clone 8 was then tested with restriction enzymes NheI, SacII and AgeI (Figure IV.32) the DNA extracted. The cloned insert, resulting in p_TARP-CRE (Figure IV.33), was sequenced, data available in Supplementary data 1.B.

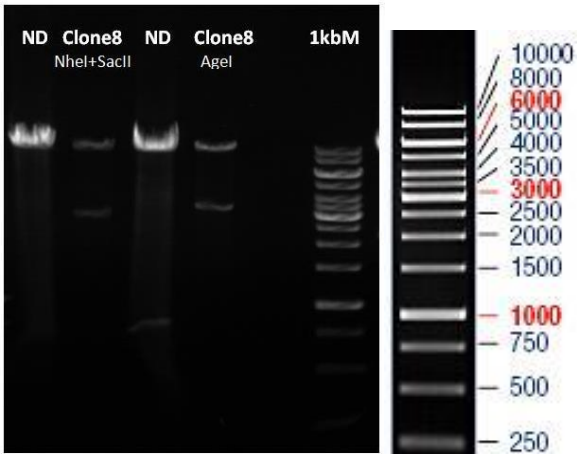

Figure IV.32 Restriction test to Clone 8. Restriction with NheI and SacII creating 10669bp and 3024bp; and digested with AgeI creating 10324bp and 3369bp. 1kbM – 1kb DNA Marker

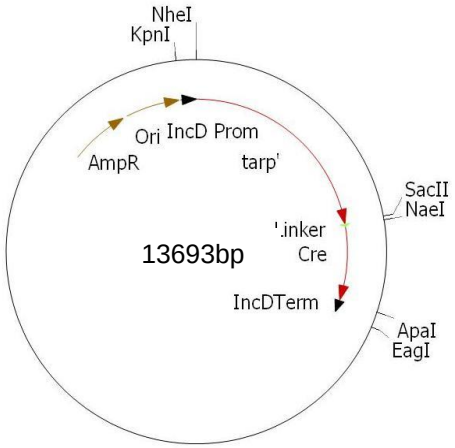

Figure IV.33 p_TARP-CRE (13693bp). Generated in Clone Manager 9.

E. Plasmids demethylation

A successful transformation of *Ctr* depends on the methylation status of the inserted DNA (Wagar *et al.*, 1992). Therefore p_101AATARP-CRE and p_TARP-CRE, were amplified in *E. coli* JM110 (*dam*$^-$ *dcm*$^-$). DNA was extracted and subsequently digested with methylation sensitive restriction enzymes, DpnI for *dam* methylation site (Figure IV.34) and EcoRII for *dcm* methylation site (Figure IV.35).

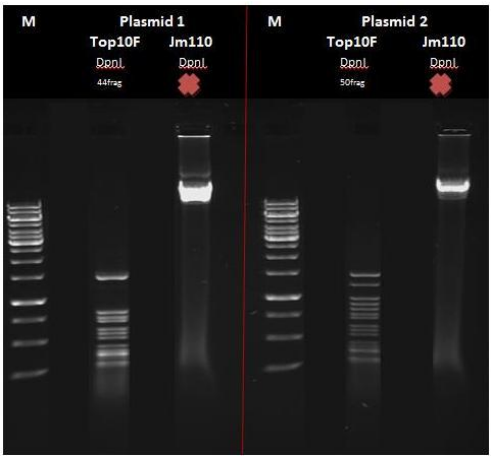

Figure IV.34 *DpnI* digestion to prove the demethylation of *dam* site methylation site. p_101AATARP-CRE (Plasmid 1) and p_TARP-CRE (Plasmid 2).

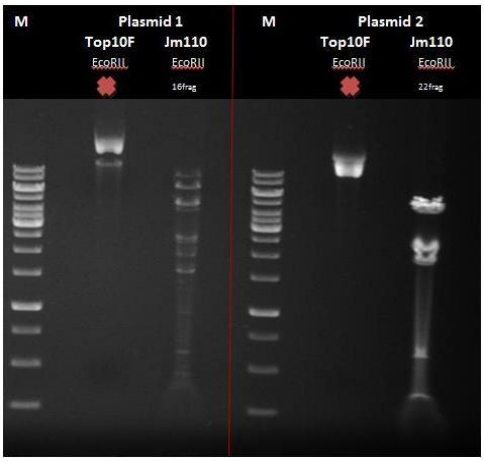

Figure IV.35 *EcoRII* digestion to prove the demethylation of *dcm* methylation site. p_101AATARP-CRE (Plasmid 1) and p_TARP-CRE (Plasmid 2).

F. Expression of CRE recombinase in *E. coli*

The correct expression of the recombinant TARP-CRE protein was tested in *E. coli* TOP10F' since the driving promoter (IncD) is functional in *Ctr* and as well in *E. coli* (Agaisse and Derré, 2013). Therefore, p_101AATARP-CRE and p_TARP-CRE were used to transform *E. coli* Top10F' and total protein lysates were analyzed by western blot (Figure IV.36).

The conjugated 101AA TARP-CRE protein (p_101AATARP-CRE) should has a calculated size of 55kDa, while the putative size of full TARP-CRE (p_TARP-CRE) is 150KDa. CRE recombinase was detected with a mouse monoclonal antibody (Amersham NA931).

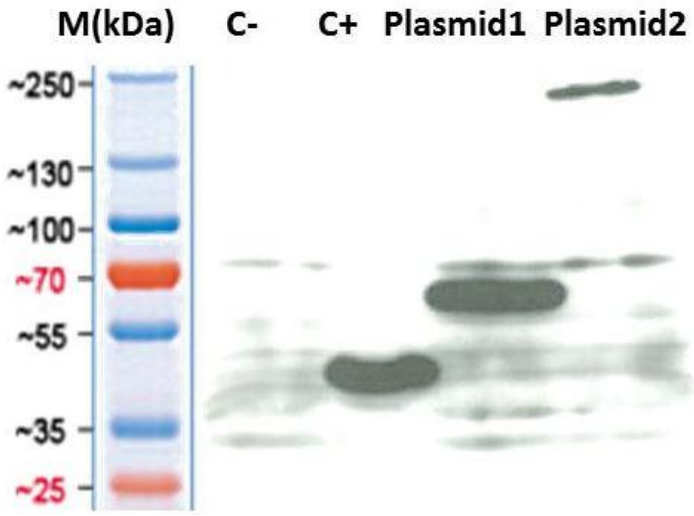

Figure IV.36 Western Blot of *E. coli* TOP10F' transformed with p_101AATARP-CRE and p_TARP-CRE using Anti-CRE recombinase Antibody. C-: plasmid without CRE; C+: plasmid with CRE recombinase conjugated with Tir protein with an expected size of 45KDa. p_101AATARP-CRE (Plasmid 1) and p_TARP-CRE (Plasmid 2).

3. Stable labeling of host cells

A. Transformation of Chlamydia trachomatis L2 with p_101AATARP-CRE and p_TARP-CRE

After the generation of p_101AATARP-CRE and p_TARP-CRE, *Ctr* L2 was independently transformed with both plasmids, using the methodology adopted for the transformation of *Ctr* L2 with p2TK2-SW2_IncDProm-mCherry-IncDTerm plasmid; Figure IV.37 for p_101AATARP-CRE and Figure IV.38 for p_TARP-CRE. As observed, at Passage 2 and Passage 3, using 0.1 U/ml of penicillin G selection, it is still possible to see both phenotypes: (1) the normal *Ctr* inclusion phenotype (red arrows) and (2) the persistence phenotype (blue arrows) described by Skilton and Lambden (Lambden *et al.*,

2006; Skilton *et al*., 2009) for the non-transformed *Ctr* L2, due to its non-resistance to penicillin G. Nevertheless, on Passage 5 and Passage 6 it is already possible to see almost only normal inclusions phenotypes what can lead to the conclusion that the present inclusions are derived from transformed *Ctr* L2 with p_101AATARP-CRE and p_TARP-CRE, respectively.

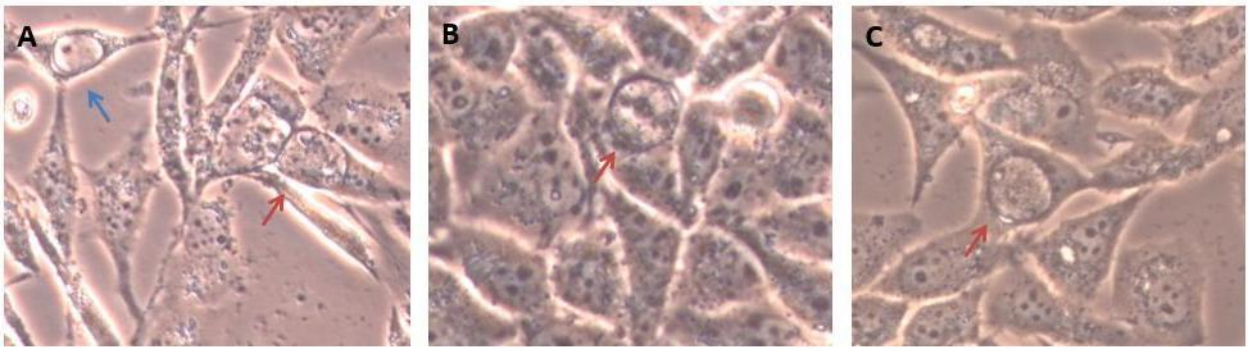

Figure IV.37 HeLa229 infected with p_101AATARP-CRE . A- P2 Day3 – 0.1 U/ml penicillin G; B- P5 Day 2 - 0.1 U/ml penicillin G; C- P6 Day3 – 0.1 U/ml penicillin G. Blue arrows show the vacuolar inclusions and the red arrows the normal inclusions (transformed *Ctr*).

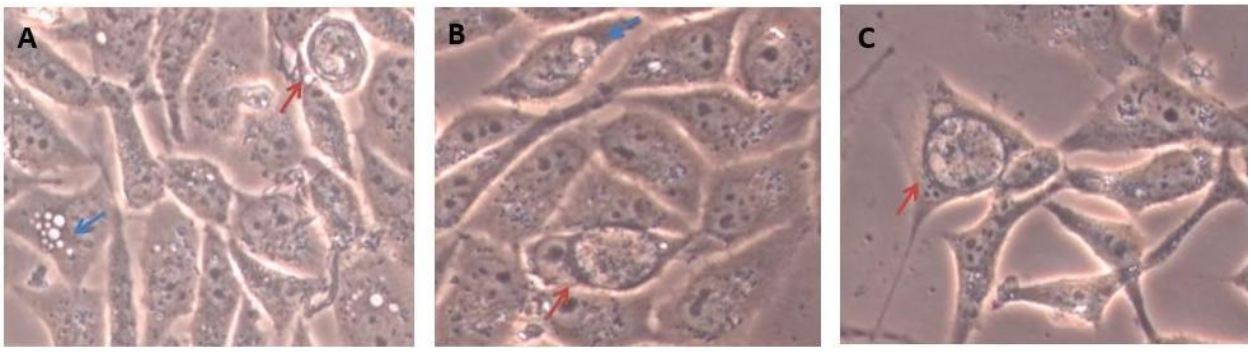

Figure IV.38 HeLa229 infected with p_TARP-CRE. A- P2 Day3 – 0.1 U/ml penicillin G; B- P5 Day2 – 0.1U/ml penicillin G; C- P6 Day2 – 0.1 U/ml penicillin G. Blue arrows show the vacuolar inclusions and the red arrows the normal inclusions (transformed *Ctr*).

B. Labeling of reporter cell line

The lysates of the transformed *Ctr* L2 with p_101AATARP-CRE and p_TARP-CRE were used to infect HeLa229_mT/mG (reporter cell line) described in the methodology (Method 1). For this step, infected cells were regularly monitored at the microscope under fluorescent light as shown in Figure IV.39 for p_101AATARP-CRE and Figure IV.40 for p_TARP-CRE.

Both Figure IV.39-B and C show that none of the cells underwent conversion from red to green fluorescence after the infection. The arrow in Figure IV.40-A points to transformed inclusions but also this cell do not show any red to green in Figure IV.40-B and C (arrow).

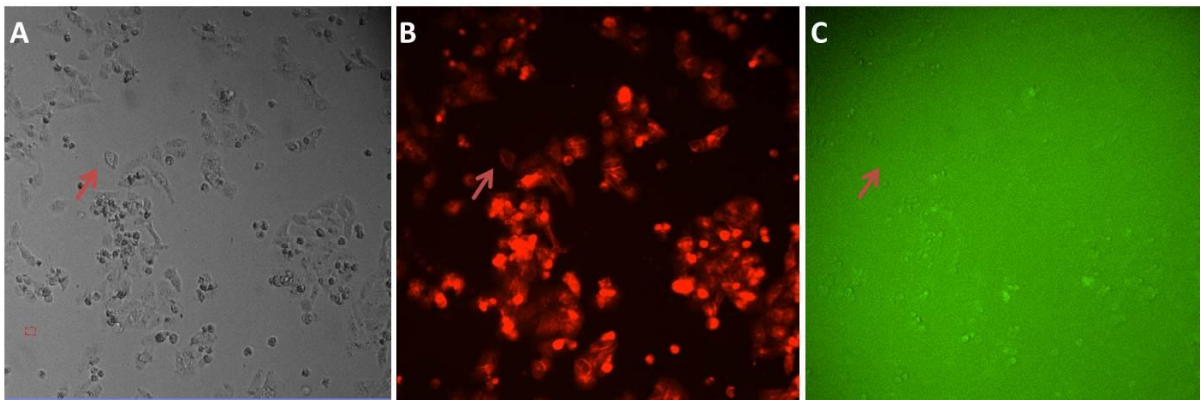

Figure IV.39 HeLa229_mT/mG infected with p_101AATARP-CRE . P5 Day2 with 0.1 U/mL of penicillin G selection. A-Brightfield; B- Cy3 filter; C- GFP filter. Acquired by live cell imaging with different exposure times.

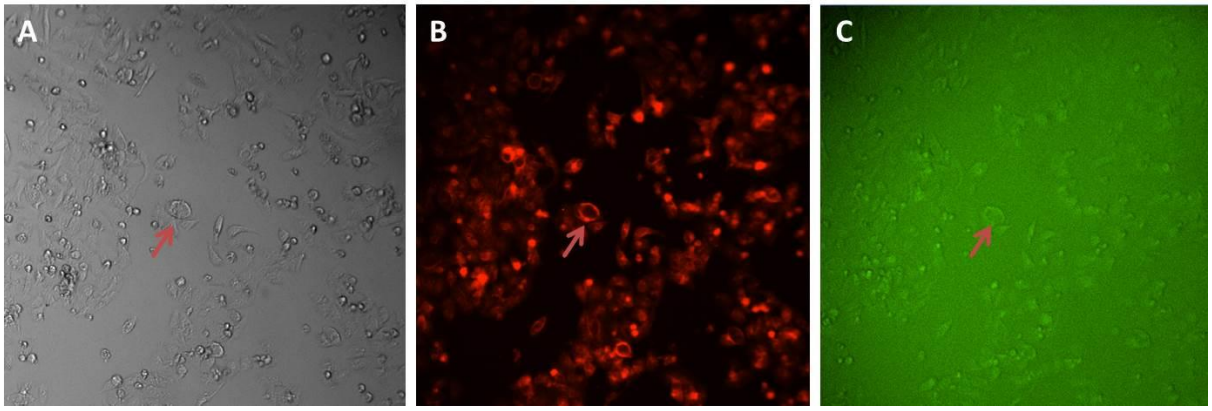

Figure IV.40 HeLa229_mT/mG infected with p_TARP-CRE. P5 Day2 with 0.1 U/mL of penicillin G selection. A-Brightfield; B- Cy3 filter; C- GFP filter. Acquired by live cell imaging with different exposure times.

Discussion

As the aim of this project was to generate a system to label permanently *Ctr* infected cells, for its purpose *Ctr* was transformed with a shuttle plasmid coding a chlamydial protein (TARP) fused with CRE recombinase and a reporter cell line sensitive to CRE recombinase activity was infected. In such a system the label of the infected cells will be irreversible and hereditary, becoming the cells detectable during the infection process and after the infection is cleared.

1. Fate of Chlamydia trachomatis infected cells

Intracellular bacteria that reside within a vacuole have the additional challenge of needing to traverse two membranes to successfully escape the host cell. For *Chlamydia*, after infection, the exit can occur by two distinct and independent processes, cellular lysis and a packaged release called extrusion (Hybiske and Stephens, 2007).

Chlamydia is also identified as able to establish asymptomatic, persistent infections by several mechanisms, including antibiotic resistance, immune evasion, and apoptosis suppression (Mpiga and Ravaoarinoro, 2006). *In vivo* the use of beta lactam antibiotics (as penicillin, for instance) in the developmental of the chlamydial cycle retards the transition to EB, resulting in aberrant RB formation where the RBs become enlarged and the developmental cycle enters a persistent, non-infectious state by blocking peptidoglycan biosynthesis through binding with penicillin binding proteins (Mpiga and Ravaoarinoro, 2006; Skilton *et al.*, 2009).

In the specific case of *Ctr*, it is reported that it modulates host cell function in ways that convey benefits to the pathogen but have severe consequences for the fate of host cells. *Ctr* induces ROS production to support its own growth and, as a result, induces the formation of DSB (Double Strand Breaks); *Ctr* also inhibits subsequent downstream repair and cell-cycle checkpoint activation that typically follow DSB induction (Chumduri *et al.*, 2013). This deregulation of host cell signaling and perturbations to the host cell chromatin lead to the enforced survival of damaged host cells, which is likely to predispose them to transformation and remain so if the pathogen disappears (Lax and Thomas, 2002; O'Driscoll and Jeggo, 2006). Therefore, during chronic infection, the infected cells undergo long term metabolic genomic stress and, as hypothesized by Chumduri and colleagues, that epigenetic modifications induced by infection are a primary mechanism that could contribute to heritable changes in host cells like the accumulation of DNA damage mutations (Chumduri *et al.*, 2013), what needs to be proven *in vivo*.

There are increasing evidences that some bacteria can contribute to specific stages in cancer development, particularly in chronic infections (Mager, 2006). These pathogens can be involved in the

hypothesis that bacterial molecules are necessary for the initiation but not the maintenance of cellular transformation (Kuper *et al*., 2000). The identification of the damaged cells that have been in contact with the pathogen could help to determine and describe the link between the pathogen and further transformations, like cancer; as the case of *Helicobater pylori* (Lax and Thomas, 2002). However, the mechanisms by which bacteria contribute to cancer formation are complex and not irrefutable proven (Vogelmann and Amieva, 2007). In the case of *Ctr*, recent evidences correlates it with cervical and ovarian cancer (Arnheim Dahlström *et al*., 2011; Koskela *et al*., 2000; Shanmughapriya *et al*., 2012). Valdivia and colleagues reported that *Ctr* infections significantly impact the cell cycle of infected cells, with evidence for cleavage of the mitotic cyclin and delay in cytokinesis and centrosomes supernumerary. These can lead to genomic instability, which in conjunction with the strong anti-apoptotic effect of *chlamydial* infection may explain the epidemiological association between infections and cervical and ovarian cancers (Chumduri *et al.*, 2013; Valdivia, 2008). However, the link between *Chlamydia* and cancer and the oncogenic mechanisms are not proved yet.

Thereby, it is required to develop a tool that can genetically label the cells that were once infected with *Ctr*, in order to study and prove if the mutations that occur while *Ctr* infection remain and once it is cleared; to clarify and link directly the various aspects that were pointed, as: what stress exactly are the cells exposed after the infection, is this stress leaving an “imprint” on the cells, is the progeny affected by these mutations, is cancer mutations correlated with it or not, between other possible relevant biological features.

2. A genetic stable cell labeling tool primed by the infection

In order to construct such a tool for the genetic label of *Ctr* infected cells, it will imply the use different components as a chlamydial secreted protein, as a vector for CRE, and a shuttle vector with a chlamydial promoter that will allow the expression of CRE in the infected cells.

To select the suitable chlamydial secreted protein that delivers the CRE recombinase in the host cell, candidates like Inc proteins, CPAF (*Chlamydia* Protease-like Activity Factor) and TARP were considered; the latter was chosen because is extensively described in the literature to be involved in the early cycle of *Chlamydia* during the first hours of infection (Fields *et al*., 2003; Mehlitz *et al*., 2010; Valdivia, 2008). The advantage of using an early expressed protein for this system is that the fused protein TARP-CRE is transcribed contemporary with its chaperon Slc1 (Brinkworth *et al.*, 2011) in the first moments of infection to the host cell.

The promoter driving the expression of the recombinant TARP-CRE, corresponds to a sequence located in the intergenic region upstream (IncD Prom) and downstream (IncD Term) of the incDEFG operon (Agaisse and Derré, 2013). The choice of the promoter and terminator regions of the incDEFG operon relies on the fact that they are expressed early and throughout the developmental cycle (Agaisse and Derré, 2013; Scidmore-carlson *et al*., 1999). This expression pattern is essential for the purpose of the project since if TARP-CRE fusion protein will be expressed early and through all infection cycle, that will maximize the chances of efficient induction of the reporter cell line.

The shuttle plasmid was provided by Agaisse and Derré (Agaisse and Derré, 2013), Figure IV.4. The choice of this plasmid relies in the fact that it was already reported a successful genetic transformation of *Ctr* L2 using this vector. This plasmid has a suitable *Ctr* promoter and terminator (IncD) an antibiotic resistance and an origin of replication that works both in *E. Coli* and in *Ctr*.

Two plasmids were constructed, p_101AATARP-CRE with the first 101AA N-terminal of TARP and p_TARP-CRE with the full TARP. 101AA TARP was chosen as a secretion signal to fuse with CRE recombinase because although normally the Slc1 chaperon binds to the first 200 N-Terminal aminoacids of TARP (Brinkworth *et al*., 2011), Pais and colleagues showed that the 100 N-terminal aminoacids of TARP are sufficient for the biding of Slc1 (Pais *et al.*, 2013). As these are the necessary aminoacids to bind to the chaperon, it is hypothesized that the fused protein will be trippingly secreted to the host cell. The cloning of p_TARP-CRE, with the full sequence of TARP to maximize the chances of secretion if the 101AA N-terminal TARP are not enough; this cloning step was more complex than p_101AATARP-CRE , mainly because of the bigger size of the plasmid, which decreases the *E. coli* transformation efficiency (Chan *et al*., 2002).

3. Functionality of the system

The described system hypothesizes that transformed *Ctr* infection can trigger a stable labeling of reporter host cells using p_101AATARP-CRE (101AA TARP fused with CRE recombinase) and p_TARP-CRE (full TARP fused with CRE recombinase), in order to study the fate of these infected cells. To achieve this it was necessary to establish a protocol for the genetic transformation of *Ctr* containing CRE to be delivered to the host. As a consequence, as Muzumdar and colleagues described, if a signal, such like CRE, is delivered to a reporter cell line sensitive to CRE, as a result the infected cells will be label in a ubiquitous and permanently way, enabling their study (Muzumdar *et al*., 2007).

A. Expression of p_101AATarp-CRE and p_TARP-CRE in E. coli

p2TK2-SW2_IncDProm-IncDTerm plasmid has an *E. coli* origin of replication (Agaisse and Derré, 2013), therefore because this plasmid was the backbone used for the generation of p_101AATARP-CRE and p_TARP-CRE, the constructed plasmids should be as well. Subsequently to the generation of both plasmids, the activity of the promoter was tested in *E. coli* using an anti-CRE recombinase antibody that detects the expression of the recombinant proteins TARP-CRE (Figure IV.36). Both p_101AATARP-CRE and p_TARP-CRE, show the expression of the respective encoded recombinant protein of 55 kDa and 150 kDa, respectively. This experiment proves that p_101AATARP-CRE and p_TARP-CRE are functional (in *E. coli*) and that the construct was correctly generated.

B. Genetic Transformation of Ctr L2 and Ctr D

In order to track *Ctr* infected cells, it was necessary to establish a protocol for *Ctr* L2 and *Ctr* D transformation. The genetic transformation of *Ctr* was not reported until three years ago, as this pathogen is considered a difficult organism to manipulate (Rockey, 2011). The genetic transformation was described in some papers since but only for *Ctr* L2 (Agaisse and Derré, 2013; Bauler and Hackstadt, 2014; Song *et al.*, 2013; Wang et al., 2011). The attempt to transform *Ctr* D for the first time is relevant because this is one of the genital serovars (D-K) known to be causative agents of the sexually transmitted diseases, accounting for up to 60 to 70% of urogenital infections (Kessler *et al.*, 2012; Singh *et al*.,2003). Transforming *Ctr* with a fluorescent protein allow more detailed studies *in vitro* and *in vivo* and using the established protocol can also be used for a different via of research, like developmental cycle studies, protein-protein interactions, etc., what can be crucial for a better understanding of the chlamydial infection.

As described in the results (1.B), the plasmid used for the genetic transformation of *Ctr*, as well as the backbone for the cloning, was provided by Agaisse and Derré (Agaisse and Derré, 2013), Figure IV.4. p2TK2-SW2_IncDProm-mCherry-IncDTerm shuttle plasmid contains the chosen chlamydial promoter and terminator (IncD) and the fluorescent protein mCherry. It is important to refer that *Ctr* is not methylated *in vivo*, its DNA appears to have demethylated *dam* sites and only low level *dcm* methylation, thus plasmid methylation decreases the transformation efficiency (Wagar *et al.*, 1992). Therefore, use of proper demethylated plasmid is crucial for *Ctr* genetic manipulation. For this reason the shuttle plasmid was amplified in a *dam dcm* deficient *E. coli* strain (*E. coli* JM110) and checked using methylation sensitive restriction enzymes; DpnI that only cleaves *dam* methylated DNA and EcoRII that does not cleave *dcm* methylated DNA. Thus, as shown in Figure IV.5, the shuttle plasmid, demethylated, was not digested by DpnI and it was digested by EcoRII creating 7 fragments, proving the demethylation of the plasmid.

To transform *Ctr* three protocols were chosen as methodological references that reported transformation in *Ctr* L2, the conjunction of details reported on each of them and the practice acquired during their performing work to create a new protocol (Method 6) that had a positive outcome transformation. Due to the different life cycle of *Ctr* D, compared to *Ctr* L2, the days of incubation between passages were longer (increasing of 24-36 h).

p2TK2-SW2_IncDProm-mCherry-IncDTerm is resistant to ampicillin (Agaisse and Derré, 2013), which belongs to the penicillin group (Raynor, 1997), allowing the selection of transformed *Ctr* L2 and D. The selection follows the principle that subsequently to the lysis or extrusion of the infected cells, the untransformed *Chlamydia*, present as non-infectious RBs, fail to passage. Thus only transformed EBs and a diminishing number of carryover untransformed EBs infect new host cells, which are easily detectable by phase contrast microscopy (Skilton *et al.*, 2009; Wang *et al.*, 2011). The phenotype between the transformed and non-transformed inclusions is visible because for *Ctr* sensitive to penicillin (non-transformed), the prolonged exposure to penicillin leads to the enlargement of the RBs and subsequent expansion of the inclusion (Lambden *et al.*, 2006; Skilton *et al.*, 2009). Therefore, as seen in Figure IV.6 and Figure IV.8 for *Ctr* L2 and *Ctr* D, respectively, it is possible to recognize the inclusions of transformed *Ctr* because they present the normal phenotype for an inclusion (red arrows) and the non-transformed ones because they present the persistence phenotype (Lambden *et al.*, 2006; Skilton *et al.*, 2009). The recovery increases with each passage, until only transformed *Ctr* remains.

Concerning the stock preparation, Figure IV.10 and Figure IV.13, the percentage of transformed *Ctr* L2 is 20-30% and for *Ctr* D is 10-20%. The sizes of inclusions formed when compared with the inclusions formed by the WT *Ctr* L2, 24 h.p.i., are the expected size and similar between them, Figure IV.12. However for transformed *Ctr* D the sizes are the expected as compared with the WT (Figure IV.15) but, on the other hand, the inclusions formed by the non-transformed *Ctr* D (80-90% of the stock) present a bigger size. This can be clarified with repetitions of the experiment, but it should be taken in account that this was performed in P0 when the ratio transformed:non-transformed is low and the big size of the non-transformed *Ctr* D could be a result of a fusion of non-transformed inclusions because at high multiplicities of infection, multiple inclusions fuse into a single inclusion (Richards *et al*., 2013). However, this non-transformed inclusions are not resistant to penicillin G, thus when repassaging it with penicillin G selection they will not be part of the infection anymore.

The established protocol for the transformation of *Ctr* L2 with p2TK2-SW2_IncDProm-mCherry-IncDTerm (Method 6), was used to transform *Ctr* with p_101AATARP-CRE and p_TARP-CRE. LGV isolates are a popular choice for laboratory work as they usually grow faster and have less compact inclusions (Wang *et al*., 2013a), thus *Ctr* L2 was the serovar chosen to start with the stable labeling of host cells; in parallel *Ctr* D was being manipulated to perform further studies. The transformation was successful for both plasmids as shown in Figure IV.37 and Figure IV.38 due to the normal inclusions with penicillin G selection. Nevertheless, repetition of this experiments for both serovars and as well the

performance of a biological assay, as the ability to synthesize and accumulate glycogen within inclusions, or by comparing expression of different proteins, etc. (Wang *et al.*, 2011), will bring clearer results on the similarities of the transformed *Ctr* L2 with the WT.

C. Stable labeling of a reporter cell line

The chosen reporter cell line to use within this study was HeLa229_mT/mG (Toelle *et al*., unpublished), which expresses a membrane-targeted tandem dimer Tomato (mT) prior to CRE-mediated excision and membrane-targeted green fluorescent protein (mG) after excision (Livet *et al*., 2007; Muzumdar *et al*., 2007; Smith, 2011). The double-fluorescent nature of HeLa229_mT/mG provides several useful features using CRE recombinase; which must be inserted downstream of a promoter to drive its expression, and is the choice of that promoter that primarily defines the specificity of CRE expression (Smith, 2011), in this case the chlamydial IncD Promoter.

The reporter cell line, HeLa229_mT/mG, has the advantage of allowing live visualization and ubiquitously labeling of cells that underwent recombination (green) or not (red). This cell line also permits lineage tracing because the changes occurring with CRE-mediated are permanent excisions of the mTomato sequence and thereby transmitted to their offspring (Muzumdar *et al*., 2007). There are other reporters published in literature like LacZ or Rosa26 LSL GFP. With this reporter the detection of b-galactosidase activity requires fixation or hypotonic permeabilization of cells, excluding all subsequent studies if viable, physically un-manipulated cells are needed. For Rosa26 LSL GFP and its variants, as live markers have effectively overcome this disadvantage of LacZ but the brightness of when expressed is insufficient to allow a truly complete separation of negative and positive cell populations due to its low tissue penetration and high tissue background fluorescence what will make impossible to accurately assess their respective developmental potential and to rigorously discriminate between potentially alternative fates (Luche *et al*., 2007).

After the transformation of *Ctr* L2 with p_101AATARP-CRE and p_TARP-CRE (Results 2.A), the transformed *Ctr* were used to infected the reporter cell line HeLa229_mT/mG as presented in Results 2.B. As it is possible to observe in Figure IV.39 and Figure IV.40 for p_101AATARP-CRE and p_TARP-CRE, respectively, inclusions are present in HeLa229_mT/mG cells in the brightfield, but when observed under fluorescence light, it is only possible to see red fluorescence cells and no green fluorescence. The lack of shift in the fluorescence could mean one of three things: (1) *Ctr* L2 is not transformed with p_101AATARP-CRE or p_TARP-CRE, (2) *Ctr* L2 is transformed but the translocation of CRE recombinase is not occurring throw the T3SS or (3) the CRE recombinase activity did not occur in host cell. Any of these three reasons could be responsible for the presented results and each one should be monitor and prove to work or not in future experiments. Thereby, in order to improve this experiment, it should be repeated for a longer time frame with increasing penicillin G selection and later performance of

a Western blotting against CRE for the lysates of *Ctr* infected cells, or an immunostaining against CRE or by a PCR reaction amplifying CRE to prove more effectively that *Ctr* is transformed indeed. Also, the efficiency of this model is known to be low using the full CRE, from parallel on-going projects with other pathogens, suggesting that it can be also a matter of probability that no green cells (expressing mG) were observed in the reporter cell line.

Considering the present results, it is necessary to consider that the bottle-neck of this model is the translocation of CRE recombinase to the host cell by T3SS, which is hampered by the unfolding for the T3SS secretion and posterior folding of the protein (Cornelis, 2006), protein abundance and proximity to the secretion apparatus (Grynberg and Godzik, 2009) between other features that influences T3SS activity that might be implicated as well. Lastly, it should also be considered that it is necessary the translocation of four CRE recombinases for the recombination activity to occur in the cell, due to its function as tetramer (Guo *et al*., 1997).

4. Conclusion and Future perspectives

In conclusion, being the objective of this project the generation of a tool for lineage tracing of *Ctr* infected cells in order to study its future, the aims proposed were fulfilled. The establishment of the genetic transformation protocol for *Ctr* L2 and *Ctr* D was successful and generation of two plasmids expressing CRE recombinase fused with two different secretions signals of *Ctr* (101AA TARP and the full TARP), that express CRE correctly were also accomplished. Finally, this system was tested for the first time in a reporter cell line (HeLa229_mT/mG) but without irrefutable postivite results. In the future, it is important to perform and repeat some experiments that will check the successful transformation of *Ctr* L2 with p_101AATARP-CRE and p_TARP-CRE and translocation of CRE, for a better understanding of the lack of red fluorescent HeLa229_mT/mG cells in the observations. An improvement in the results could also be achieved by developing the use of different methodologies for the stable labeling of host cells, such as the use of Split-CRE recombinase instead of the full CRE recombinase or the use of a TEV protease combined with CRE recombinase (TEV-CRE System), linked to the inner cell membrane of the host. These two alternatives could help the improvement the system because for Split-CRE recombinase the length of the fused protein will be smaller when folded what will improve the secretion through T3SS. TEV-CRE system, can also benefit the labeling model, because the system is not dependent on the translocation or not of CRE recombinase through the T3SS, from the pathogen to the host; it´s only necessary the trigger signal that can be a common chlamydial effector.

This project, still ongoing, has set the basis for further developments of the primed genetic labelling of infected cells using *Ctr*. However, this new system is under improvement and it will be used as a part of a future PhD project that will develop and improve the presented results and as well trying possible alternatives, in order to perform the stable labeling of *Ctr* infected cells allowing its tracing and study.

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VII. Supplementary data

1. Sequencing Data

A. p_101AATARP-CRE

Backbone is p2TK2-SW2 described in Agaisse and Derré (Agaisse and Derré, 2013)

Insert (101AATARP-CRE)

GCTAGCATGACGAATTCTATATCAGGTGATCAACCTACTGTTACAACTTTTACATCATCAACCACTTCGGCATCAGGTGCTTCCGGATCTCTGGGAGCTTCTTCTGTATCTACT
ACCGCAAACGCTACAGTTACACAAACAGCAAACGCAACAAATTCAGCGGCTACATCTTCTATCCAAACGACTGGAGAGACTGTAGTAAACTATACGAATTCAGCCTCCGCCC
CCACTGTAACTGTATCGACCTCCTCTTCTTCCACACAAGCCACAGCCACTTCGAATAAAACTTCCCAAGCCGTTGCTGGAAAACCGCGGGCCAGCCCTAGCAACCCTGGCG
CCAGCAACGGCAGCGCCGGCATGCCCAAGAAGAAGAGGAAGGTGTCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAACGAGTGATGAGGTTC
GCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTGCAAGTTGAATAACC
GGAAATGGTTTCCCGCAGAACCTGAAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGCCAGCTAAACATGCT
TCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCT
CTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTT
ACGTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGCAGGTGTA
GAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGT
GTTGCCGCGCCATCTGCCACCAGCCAGCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATAC
CTGGCCTGGTCTGGACACAGTGCCCGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGACCAATGTAAATAT
TGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTGCGCCTGCTGGAAGATGGCGATTAGGTCGAGGGCCCGG

B. p_TARP-CRE

Backbone is p2TK2-SW2 described in Agaisse and Derré (Agaisse and Derré, 2013)

Insert (TARP-CRE)

CTAGCATGACGAATTCTATATCAGGTGATCAACCTACTGTTACAACTTTTACATCATCAACCACTTCGGCATCAGGTGCTTCCGGATCTCTGGGAGCTTCTTCTGTATCTACTA
CCGCAAACGCTACAGTTACACAAACAGCAAACGCAACAAATTCAGCGGCTACATCTTCTATCCAAACGACTGGAGAGACTGTAGTAAACTATACGAATTCAGCCTCCGCCCC
CACTGTAACTGTATCGACCTCCTCTTCTTCCACACAAGCCACAGCCACTTCGAATAAAACTTCCCAAGCCGTTGCTGGAAAAATCACTTCTCCAGATACTTCAGAAAGCTCAG
AAACTAGCTCTACCTCATCAAGCGATCATATCCCTAGCGATTACGAGCCGATAAGCACTACTGAAAATATTTATGAGAATATTTATGAGAGTATTGATGACTCTAGCACAAGTG
GCCCAGAAAATACGAGTGGTGGTGCAGCAGCACTCAATTCTCTAAGAGGCTCCTCCTACAGCAATTATGACGATGCTGCTGCTGATTACGAGCCGATAAGCACTACTGAAAA
TATTTATGAGAGTATTGATGACTCTAGCACAAGTGACCCAGAAAATACGAGTGGTGGTGCAGCAGCACTCAATTCTCTAAGAGGCTCCTCCTACAGCAATTATGACGATGCTG
CTGCTGATTACGAGCCGATAAGCACTACTGAAAATATTTATGAGAATATTTATGAGAGTATTGATGACTCTAGCACAAGTGGCCCAGAAAATACGAGTGGTGGTGCAGCAGCA
CTCAATTCTCTAAGAGGCTCCTCCTACAGCAATTATGACGATGCTGCTGCTGATTACGAGCCGATAAGCACTACTGAAAATATTTATGAGAGTATTGATGACTCTAGCACAAG
TGACCCAGAAAATACGAGTGGTGGTGCAGCAGCAGCACTCAATTCTCTAAGAGGCTCCTCCTACAGCAATTATGACGATGCTGCTGCTGATTACGAGCCGATAAGCACTACT
GAAAATATTTATGAGAGTATTGATGACTCTAGCACAAGTGACCCAGAAAATACGAGTGGTGGTGCAGCAGCACTCAATTCTCTAAGAGGCTCCTCCTACAGCAATTATGACGA
TGCTGCTGCTGATTACGAGCCGATAAGCACTACTGAAAATATTTATGAGAATATTTATGAGAGTATTGATGGCTCTAGCACAAGTGACCCAGAAAATACGAGTGGTGGTGCAG
CAGCAGCACTCAATTCTCTAAGAGGCTCCTCCTACACAACAGGGCCTCGTAACGAGGGTGTATTCGGCCCTGGACCGGAAGGACTACCAGACATGTCTCTTCCTTCATACG
ATCCTACAAATAAAACCTCGTTATTGACTTTCCTCTCCAACCCTCATGTAAAGTCGAAAATGCTTGAAAACTCGGGGCATTTCGTCTTCATTGATACAGATAGAAGTAGTTTCA
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CAGGGTTTAACAAATTCTCTGGTGACTGGGACAGTCGTGTAGAACCTATGATGTCAGCCAAAGCTGGAGTGGCCAGCGGAGGCAATCTTCCCAATACAGTGATTATCAATAA
TAAATTCAAAACTTGCGTTGCTTATGGTCCTTGGAATAGCCGGGAAGCAAGTTCTGGTTATACACCTTCTGCTTGGAGACGTGGTCATCAAGTAAATTTTGGAGAAATTTTTGA
GAAAGCCAACGACTTTAATAAAATCAACTGGGGAACTCAAGCCGGGCCTAGTAGCGAAGACGATGGCATTTCCTTCTCCAATGAAACTCCTGGAGCTGGTCCTGCAGCTGCT
CCATCACCAACGCCATCCTCTATTCCTGTCATCAATGTCAATGTCAACGTTGGCGGAACTAATGTGAATATTAGAGATACGAATGTCAACACGACTAACACCACACCAACAAC
TCAATCTACAGACGCCTCTACAGATACAAGCGATATCGATAACATAAATACCAACAACCAAACTGATGATATCAATACGACAGACAAAGACTCTGACGGAGCTGGTGGAGTCA
ATGGCGATATATCCGAAACAGAATCCTCTTCTGGAGATGATTCAGGAAGTGTCTCTTCCTCAGAATCAGACAAGAATGCCTCTGTCGGAAATGACGGACCTGCTATGAAAGA
TATCCTTTCTGCCGTCGTAAACACCTAGACGTCGTTTACCCTGGCGACAATGGTGGTTCTACAGAAGGACCTCTCCAAGCTAACCAAACTCTTGGAGATATCGTCCAGGAT
ATGGAAACAACAGGGACATCCCAAGAAACCGTTGTATCCCCATGGAAAGGAAGCACTTCTTCAACGGGATCAGCAGGAGGAAGTGGTAGCGTACAAACACTACTGCCTTCA
CCACCTCCAACCCCATCAACTACAACATTAAGAACGGGCACAGGAGCTACCACCACATCCTTGATGATGGGAGGACCAATCAAAGCTGACATAATAACAACTGGTGGCGGA
GGACGAATTCCTGGAGGAGGAACGTTAGAAAAGCTGCTCCCTCGTATACGTGCGCACTTAGACATATCCTTTGATGGGCAAGGCGATCTCGTAAGTACTGAAGAGCCTCAG
CTTGGCTCGATTGTAAACAAATTCCGCAAAGAAACTGGTTCAGGAGGAATCGTAGCTTCCGTTGAGAGTGCTCCAGGCAAGCCGGGATCTGCACAGGTCTTAACAGGTACA
GGGGGAGATAAAGGCAACCTATTCCAAGCAGCTGCCGCAGTCACCCAAGCCTTAGGAAATGTTGCAGGGAAAGTCAACCTTGCGATACAAGGCCAAAAACTATCATCCCTA
GTCAATGACGACGGGAAGGGGTCTGTTGGAAGAGATTTATTCCAAGCAGCAACCCAAACAACTCAAGCGCTAAGCTCAACTGATTGATACCGTAGGACCGCGGGCCAGCCCT
AGCAACCCTGGCGCCAGCAACGGCAGCGCCGGCATGCCCAAGAAGAAGAGGAAGGTGTCCAATTTACTGACCGTACACCAAAATTTGCCTGCATTACCGGTCGATGCAAC
GAGTGATGAGGTTCGCAAGAACCTGATGGACATGTTCAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGTGGGCGGCATGGTG
CAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCGCGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGGGC
CAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGTTTCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGT
GCAAAACAGGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTT
ATAACACCCTGTTACGTATAGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAATGTTAATCCATATTGGCAGAACGAAAACGCTGGTTAG
CACCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGGT
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GGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTGTCGGAGCCGCGCGAGATATGGCCCGCGCTGGAGTTTCAATACCGGAGATCATGCAAGCTGGTGGCTGGA
CCAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTGCGCCTGCTGGAAGATGGCGATTAGGTC

2. Master-Student Study Plan at Max Planck Institute for Infection Biology

Name/Initials: **Ana Rita Teixeira da Costa / ARC** Date: 19/02/2014
Thesis supervisor: Prof. Dr. Thomas Meyer Start of Studies: 02/09/2013
Scientific team: Benjamin Toelle, Dr. Erik Gonzalez, Dr. Francesco Bocellatto
Title of the thesis: Fate of *Chlamydia trachomatis* infected cells
University: Universidade Nova de Lisboa (UNL)
Faculty: Faculdade de Ciencias e Tecnologia (FCT)
Faculty supervisor: Dr. Pedro Viana Baptista

Projects

Project No.	Title/short description	Status
MB079	Fate of *Chlamydia trachomatis* infected cells (Cells that were previously in contact with *Chlamydia trachomatis* can be labelled using the double-fluorescent CRE system. Goal: Label *Chlamydia trachomatis* (serovar L2 and D) previously infected cells.	Active

Seminar and course participation

Date	Type/subject	Remarks
10/10/2013	BLSC Seminar - Jacques Neefjes (The Netherlands cancer Institute)	"How Salmonella causes cancer"
16/01/2014	BLSC Seminar - Maria Manuel Mota (Universidade de Lisboa, Portugal)	"Host-Plasmodium interactions: Understanding to intervene"
13/03/2014	Karina Xavier (Instituto Gulbenkian de Ciência, Oeiras, Portugal)	"Manipulating interspecies quorum sensing in bacterial consortia"

Internal presentations

Date	Type/subject	Remarks
24/10/2013	Project presentation and discussion	Within the Genetic System Club
10/12/2013	Project updates and Review of Literature	Within the *Chlamydia* Club
23/01/2014	Project updates presentation	Within the Departmental Retreat
24/01/2014	Review of *Chlamydia* T3SS	Within the Departmental Retreat
27/02/2014	Crispr-Cas System Review	Within the Genetic System Club
31/03/2014	Tips and Tricks ("i-lab Apps")	Within the Departmental Seminar
2/06/2014	Project Final Presentation	Within the Departmental Seminar

Regular/occasional participation in departmental seminars, discussion clubs etc.

Period (from-to)	Type/subject	Remarks (frequency: occ./reg./etc.)
September – Present	*Chlamydia* Club	Bi-weekly
September – Present	Genetic System Club	Bi-weekly
September – Present	Cells and Cancer Club	Bi-weekly

General methods deposited

No.	Title	Date
ARC003.A	Infection of cells with *Chlamydia trachomatis* (serovar L2 and D)	04.09.2013
ARC003.E	Immunofluorescence Staining (General method)	17.09.2013
ARC003.D	Transformation of *Chlamydia trachomatis*	11.10.2013
ARC003.H	TA Cloning	11.11.2013
ARC003.N	Molecular Cloning (General method)	11.02.2014